GENETIC ANALYSIS OF EMBRYONAL CARCINOMA CELLS

AUSTIN GERARD SMITH

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ABSTRACT

Embryonal carcinoma (EC) cells closely resemble early embryonic stem cells. They can be induced to differentiate by treatment with retinoic acid. The interactions of parental and variant EC clones with retinoic acid and their expression of cellular retinoic acid binding protein (CRABP) have been investigated. The first variant, PTmr0, is a previously isolated clone resistant to the inhibitory effect of retinoic acid on metabolic cooperation. It is demonstrated that PTmr0 retains responsiveness to the induction of differentiation by retinoic acid, though with a reduced sensitivity compared to its parental line PSA4TG12. It is unclear whether this altered sensitivity is causally related to the variant communication phenotype. A second previously isolated variant, PT2md1, possesses a decreased ability for metabolic cooperation. This variant also exhibits a greatly reduced capacity for differentiation. It is shown that PT2md1 does not differentiate on exposure to retinoic acid, but dies. Such mortality is highly unusual and appears to be directly related to the incapacity for differentiation. Two new variants have also been selected which are deficient in their differentiation responses to retinoic acid. One undergoes partial, reversible differentiation, whilst the other appears completely unresponsive. All the EC lines examined, both variant and parental, express CRABP.

Many pluripotent EC lines and all embryonal stem cell lines established directly from embryos (EK cells) differentiate unless they are cultured on feeder layers of growth-arrested embryonic fibroblasts. The discovery is reported that medium conditioned by incubation with Buffalo rat liver (BRL) cells can support the proliferation of homogeneous undifferentiated populations of such cells in the absence of feeders. The "Differentiation Inhibiting Activity" (DIA) released by BRL cells is fully reversible in its effects. It therefore has valuable experimental applications. DIA also inhibits the differentiation response to two other stimuli; aggregation and retinoic acid. Induced differentiation of the EC line PSA4 in the presence of DIA appears restricted to the extraembryonic endoderm lineage. The finding that a similar activity is produced by the PYS parietal endoderm cell line adds weight to the suggestion that DIA may function in embryogenesis as a selective inhibitory influence.

DIA has been partially purified. It is an acid and relatively heat-stable entity of apparent molecular weight 20-35000 by gel filtration. DIA co-purifies with an activity which is mitogenic for rat fibroblasts but it does not correspond to any of a panel of growth factors tested, both individually and in combination.

The proliferation of feeder-dependent EC cells in the absence of feeders also requires the presence of 2-mercaptoethanol. It is demonstrated that this is necessary for cell viability and is unlikely to have any direct effect on differentiation. Finally, preliminary evidence is presented that the growth of EC cells on feeders in the absence of 2-mercaptoethanol may be made possible by the junctional transfer of some essential metabolite(s).

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DECLARATION

This thesis has been composed by myself. With the exception of the help and guidance mentioned above it is entirely my own work.

A. G. Smith

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ABBREVIATIONS

AFP	alphfoetoprotein
AMP	adenosine 5'-monophosphate
CM	complete medium
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
DFMO	difluoromethylornithine
DIA	differentiation inhibiting activity
dif	differentiation
DMSO	dimethyl sulphoxide
Doxyl	4',4'-dimethlyoxazolidine-N-oxyl
EC	embryonal carcinoma
ECDGF	embryonal carcinoma-derived growth factor
EDTA	ethylenediaminotetraacetic acid
EGF	epidermal growth factor
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HMBA	hexamethylenebisacetamide
ICM	inner cell mass
IGF	insulin-like growth factor
mec	metabolic cooperation
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MSA	multiplication stimulating activity
MTANP	methylthioadenosine nucleoside phosphorylase
PBS	phosphate buffered saline, complete
PBSA	phosphate buffered saline, solution A
PDGF	platelet-derived growth factor
PMA	phorbol-12-myristate,13-acetate
SSEA-1	stage-specific embryonic antigen 1
TGF	transforming growth factor
TBS	tris-buffered saline
NRS	normal rabbit serum

CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION

Mammalian developmental biology is concerned with the processes by which a single fertilized egg cell gives rise to a multicellular organism consisting of an integrated three-dimensional array of differentiated cell types. Embryonic development proceeds via the spatially and temporally ordered determination and differentiation of populations of progenitor cells. This thesis is an attempt to elucidate some of the mechanisms controlling this process for a pluripotent population of cells in the early mouse embryo, the inner cell mass (ICM). The ICM is the forebear of all three germ layers of the embryo proper, as well as most of the extra-embryonic tissues.

Due to the practical and technical limitations of experimentation on early embryos, a model system has been employed for this study. This is the use of murine embryonal carcinoma (EC) cells as *in vitro* counterparts of pluripotent embryo cells. The former are readily propagated in culture and have been widely used by mammalian developmental biologists (reviewed by Martin, 1975,1980; Damjanov and Solter, 1979; Nicolas *et al*, 1981a).

Section 1.2. is an overview of early events in normal mouse embryogenesis. There then follows an account of EC cells and their relation to embryo cells (Section 1.3.). Differentiation of EC cells *in vitro* and the effects of chemical inducing agents are discussed in Sections 1.4. and 1.5., with particular emphasis on the role of retinoic acid and its possible mode of action. Cell lines which closely resemble EC cells can be established directly from embryos. These are known as EK cells and their properties are summarized in Section 1.6. Section 1.7. considers the potential roles of endogenous and exogenous factors in the regulation of EC cell proliferation and differentiation and the potential importance of such factors in embryogenesis. Much of the work in this study is on EC cells with variant junctional communication properties. Therefore a brief description of the biology of metabolic cooperation and some of the methodology employed in its study is presented in Section 1.8. Finally (Section 1.9.), a brief outline is given of the major areas of experimental

investigation in this thesis.

1.2. DEVELOPMENT OF THE EARLY MOUSE EMBRYO

After the human, the mouse has been the most studied of mammalian embryos. Its development proceeds in an ordered and well-documented sequence of events (Theiler, 1972). Characterisation of the ontogenetic process has largely been confined to the descriptive level, however, with the underlying mechanisms of organogenesis, morphogenesis, differentiation and cell determination remaining obscure. Only in the pre-implantation embryo has any real progress been made at the molecular level (reviewed by Adamson and Gardner, 1979; Johnson, 1981; Gardner, 1983a).

1.2.1. Development Prior to Implantation

The mouse embryo begins life with the fusion of two haploid gametes, the female ovum and the male sperm, in the uppermost loop of the oviduct known as the ampulla tubae. For 20 hours after fertilisation, the egg, enveloped by a mucoprotein membrane the zona pellucida, migrates down into the second loop of the oviduct. Towards the end of this period the male and female pronuclei fuse and enter into the first cleavage division (Theiler, 1972). Over the next 48 hours the cleavage stage embryo undergoes a further three rounds of division to generate a morula composed of approximately 16 individual cells or blastomeres (Fig.1.1). The blastomeres are roughly spherical and mutual contact between them is minimal until compaction (Adamson and Gardner, 1979). This phenomenon arises at the 8-cell stage and persists throughout the remainder of cleavage. The blastomeres come into intimate and extensive contact with one another and tight junctions form between the outside cells (Adamson and Gardner, 1979). Compaction is accompanied by reorganization of the cytoskeleton and redistribution of mitochondria and microvilli (Adamson and Gardner, 1979; Johnson, 1981).

Compaction introduces heterogeneity into the morula embryo as it allows the establishment of disparate microenvironments between inside and outside cells. This inside-outside difference leads to a progressive divergence of the cells into two populations with different fates (reviewed by Johnson, 1981; Gardner, 1983a). The outside cells develop into trophectoderm whilst the inner cells

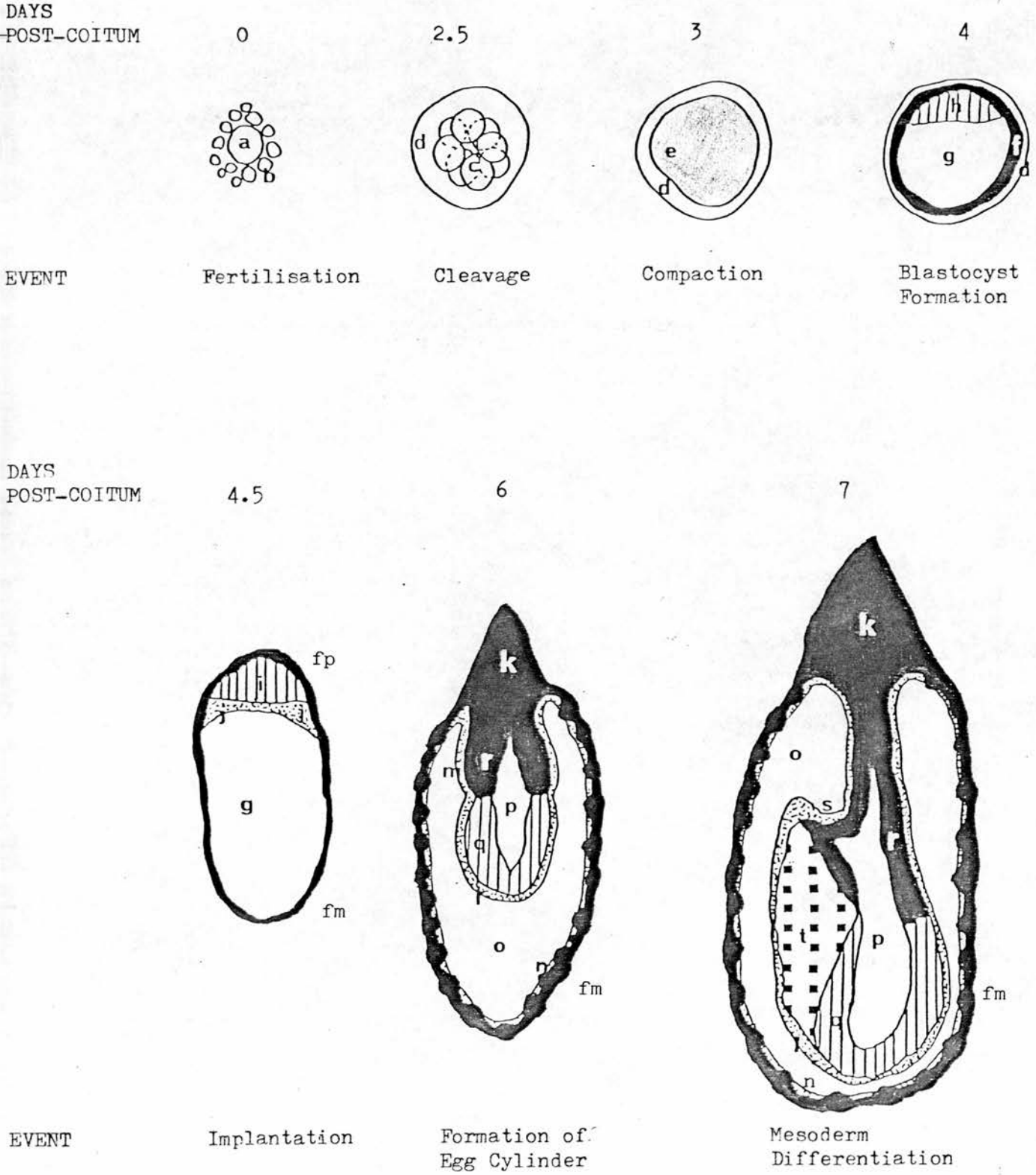
Fig.1.1 Early Development of the Mouse Embryo

Adapted from Adamson and Gardner (1979) and Gardner (1983a)

(Cell boundaries are shown only in the first two diagrams)

a: ovum
b: granulosa cells
c: 1/8 blastomere
d: zona pellucida
e: compacted blastomeres
f: trophectoderm
fp: polar trophectoderm
fm: mural trophectoderm
g: blastocoel
h: inner cell mass (ICM)
i: primitive ectoderm
j: primitive endoderm
k: ectoplacental cone
l: visceral embryonic endoderm
m: visceral extraembryonic endoderm
n: parietal endoderm
o: yolk sac cavity
p: proamniotic cavity
q: embryonic ectoderm
r: extraembryonic ectoderm
s: transverse fold
t: mesoderm

Fig 1.1. EARLY DEVELOPMENT OF THE MOUSE EMBRYO



give rise to the inner cell mass (ICM) (see Fig.1.1).

Overt cellular differentiation does not become manifest until the fourth day of development, however (Adamson and Gardner, 1979). By this time the peristaltic contractions of the oviduct have moved the embryo into the uterus. At the 35-cell stage a large eccentrically placed lumen, known as the blastocoel, develops. Around the outside of this cavity is a layer of flattened epithelial cells, the trophectoderm. This is readily distinguished from the compacted cluster of cuboidal inner cell mass cells at one pole of the developing blastocyst (Fig.1.1).

During the next 24 hours both tissues undergo further regional differentiation so that by the time of implantation (4.5 days after fertilisation) four cell populations are evident (Fig.1.1). By the end of the fourth day the blastocyst has 'hatched' from the zona pellucida and the trophoblast is in close contact with the uterine wall (Theiler, 1972). The trophectoderm develops into topographically distinct mural and polar components at this stage. The latter is situated over the ICM whilst the former surrounds the blastocoelic cavity.

As the blastocyst begins to implant, the ICM differentiates into primitive ectoderm (or epiblast) and a distinct layer of primitive endoderm cells (Enders *et al*, 1978). The latter forms at the interface of the ICM with the blastocoel. This development may be initiated by an inside-outside difference as with the earlier bidirectional differentiation of the morula (Gardner, 1983a). Primitive ectoderm cells transiently become less closely associated with one another following the delamination of endoderm (Enders *et al*, 1978). The primitive endoderm is characterised by the presence of extensive cisternae of endoplasmic reticulum filled with amorphous matrix material (Enders *et al*, 1978). The two cell types have distinct developmental fates (Gardner and Papaioannou, 1975; Gardner and Rossant, 1979; Gardner, 1983a). Primitive endoderm only gives rise to extraembryonic visceral and parietal endoderm, whilst primitive ectoderm is the forebear of the foetal soma and germ line and also of amniotic ectoderm and extraembryonic mesoderm.

1.2.2. Early Post-Implantation Development

The mouse blastocyst normally implants on the fifth day after fertilization. During the succeeding 24 hours the polar trophectoderm gives rise to an

outgrowth called the ectoplacental cone, which projects into the uterine wall (Theiler, 1972). At the same time the underlying epiblast proliferates and bulges into the blastocoelic cavity to form the egg cylinder. The primitive ectoderm cells become recompacted during this phase (Enders *et al*, 1978).

Extraembryonic ectoderm, derived from the polar trophectoderm (Gardner, 1983a), migrates into the elongating cylinder so that the latter is divided into embryonic and extraembryonic regions on day 6 (Fig.1.1). The cells of the embryonic ectoderm are generally cylindrical. They are usually separated from the irregular cuboidal cells of the extraembryonic ectoderm by a small furrow (Theiler, 1972). A central lumen appears, initially in the embryonic area but subsequently extending into the extraembryonic region. This is the proamniotic cavity (Fig.1.1).

The primitive endoderm differentiates into two topographically and morphologically distinct forms of mature extraembryonic endoderm after implantation (Theiler, 1972; Enders *et al*, 1978; Hogan and Tilly, 1981; Gardner, 1983a). Some of the endoderm cells spread onto the inner surface of the mural trophectoderm and line the entire surface of the former blastocoelic cavity. This discontinuous cell layer is the parietal endoderm. Those endoderm cells which remain in contact with the ectodermal cells of the egg cylinder mature into a continuous epithelium known as the visceral endoderm (Fig.1.1).

Along with the overlying trophoblast giant cells parietal endoderm forms the parietal yolk sac. Parietal endoderm cells share only limited intercellular contacts and assume a flattened fibroblastic form. They are separated from the trophectoderm by a basement membrane known as Reichert's membrane (Theiler, 1972; Adamson and Ayers, 1979). This thickens during subsequent development of the embryo and may act as a coarse filter which prevents access of maternal cells (Gardner, 1983a). Reichert's membrane is synthesized by the parietal endoderm, though trophoblastic components may play a role in its origin (Gardner, 1983a). Consistent with their synthetic role, parietal endoderm cells exhibit dilated profiles of rough endoplasmic reticulum filled with matrix material (Enders *et al*, 1978; Hogan and Tilly, 1981) and secrete large amounts of type IV procollagen (Adamson and Ayers, 1979), laminin (Hogan, 1980; Hogan *et al*, 1980), heparan sulphate and other sulphated glycoproteins (Hogan *et al*, 1982), all of which are incorporated into Reichert's membrane.

Parietal endodrm cells also secrete the tissue activator form of plasminogen activator (Strickland *et al*, 1976; Marotti *et al*, 1982). This can be distinguished from the urokinase form produced by mature visceral endoderm and most other embryonic tissues both immunologically and by molecular weight (Marotti *et al*, 1982). Unlike urokinase, tissue activator is stimulated by fibrin and fibrin degradation products (Ranby *et al*, 1982; Bachmann and Kuithof, 1984). It therefore exhibits non-linear dose-response characteristics and displays greater activity than urokinase in assays based on proteolysis of fibrin. Visceral endoderm is formed by those primitive endoderm cells which continue to invest the primitive ectoderm and subsequently also envelop the invaginated extraembryonic ectoderm (Fig.1.1). The mature epithelium consists of a single layer of closely apposed cuboidal cells joined by apical junction complexes (Enders *et al*, 1978). The endoderm cells reside on thin deposits of extracellular matrix similar in composition to Reichert's membrane, but they maintain intercellular contact with the underlying ectoderm via cytoplasmic projections from the latter which interrupt the basal lamina (Gardner, 1983a). At the ultrastructural level visceral endoderm cells possess only narrow profiles of endoplasmic reticulum but exhibit numerous short microvilli on their apical surfaces. They contain many apical electron-lucent vacuoles and pinocytotic vesicles (Enders *et al*, 1978; Hogan and Tilly, 1981). This morphology is suggestive of an absorptive role and the visceral endoderm can endocytose and degrade proteins and pass on the free amino acids generated to the embryo (reviewed by Gardner, 1983a). This cell layer may therefore play a key role in nutrition of the embryo. Together with the visceral mesoderm it forms the visceral yolk sac which envelopes the growing foetus. The visceral endoderm does not give rise to definitive endoderm or to any other foetal tissues (Gardner, 1983a).

Visceral endoderm is the unique source of alphafoetoprotein (AFP) in the early embryo until development of the foetal liver (Dziadek and Adamson, 1978). AFP can be detected immunologically in the 7 day embryo and this provides a reliable means of identifying visceral endoderm at this stage. However, only the embryonic portion of the endoderm layer synthesizes AFP due to an inhibitory influence of extraembryonic ectoderm (Dziadek, 1978). Moreover, other tissues adsorb AFP as post-implantation development proceeds, though this problem can be circumvented by the technique of in situ hybridisation to mRNA which identifies only the sites of synthesis of AFP (Kurkinen *et al*, 1983;

Dziadek and Andrews, 1983). Another marker of visceral endoderm is the production of transferrin (Adamson, 1982). Visceral endoderm cells also differ from parietal cells in the manufacture of basement membrane components. Unlike the latter, they synthesize fibronectin (Hogan, 1980), but they do not make significant amounts of laminin (Hogan, 1980; Howe and Solter, 1980). Visceral endoderm produces little type IV procollagen but does make type I collagen later in development (Adamson and Ayers, 1979).

Cell lineage studies (Gardner and Papaioannou, 1975; Gardner and Rossant, 1979; Gardner, 1983a) have established that parietal and visceral endoderm arise from a common founder population, the primitive endoderm. Contact with trophectoderm is not necessary for differentiation of parietal endoderm (Wiley *et al*, 1977; Enders, 1978; Gardner, 1983a) and the endoderm in the the late blastocyst is often multi-layered with the superficial cells already adopting the mature parietal form. Conversely, those cells which retain contact with the ectoderm mature into visceral endoderm. This suggests that loss of contact with the underlying ectoderm may be a sufficient condition for conversion of primitive into parietal endoderm cells (Gardner, 1983a). Consistent with this is the finding that prior to elongation of the egg cylinder, parietal cells outnumber visceral cells (Enders *et al*, 1978).

Hogan and Tilly (1981) have also presented evidence that local cell interactions modulate development of the parietal or visceral phenotype. They found that the visceral endoderm layer of the 6.5 day embryo will differentiate into parietal endoderm when cultured in contact with trophoblast rather than ectodermal cells. This presents the possibility that the differences between the two forms of extraembryonic endoderm arise from modulation of a single cell state or "transdifferentiation" (Hogan *et al*, 1983). However, it seems more likely that the visceral layer still contains some bipotential primitive endoderm cells at this early stage, which can generate either the visceral or parietal phenotype depending on local environmental factors (Gardner, 1983a).

During day 7 of gestation the proamniotic cavity expands and mesoderm appears at the posterior end of the primitive streak, establishing the embryonic axis (Theiler, 1972). The embryonic mesoderm spreads anteriorly between the ectoderm and endoderm (Fig.1.1). Both embryonic and extraembryonic mesoderm originate from the primitive ectoderm (Gardner and Rossant, 1979: Gardner, 1983a). They exhibit a random pattern of inactivation of the X

chromosome in female embryos, unlike trophoblast and extraembryonic endoderm which display preferential inactivation of the paternal X, but in common with all other derivatives of the primitive ectoderm (West, 1977).

There has been very little successful experimental investigation into later differentiation within the primitive ectoderm. However, Beddington (1982) used *in vitro* chimaeras to analyse the tissue potency of different regions of the embryonic ectoderm on day 8. The results of orthotopic and heterotopic injections showed that all regions of the ectoderm remained capable of contribution to mesoderm, both embryonic and extraembryonic, or to definitive ectoderm, depending on their localisation. This argues for the persistence of cellular lability in the embryonic ectoderm after primitive streak formation and against a rigid mosaic model of cell determination (Beddington, 1982).

At the end of day 7 the posterior end of the primitive streak bulges into the proamniotic cavity to form the posterior amniotic fold. Subsequently a cavity, the exocoelom, forms within the extraembryonic mesoderm, the amnion closes over, and by day 8 the egg cylinder contains three separate cavities; amniotic cavity, exocoelom and ectoplacental cleft (Theiler, 1972).

The germ line does not become apparent as a distinct lineage in the mouse embryo until after gastrulation. Primordial germ cells can be recognised for the first time on day 9 within the epithelium of the hind-gut (Theiler, 1972). They migrate to a pair of structures called genital ridges which subsequently give rise to the gonads. Sexual differentiation is histologically detectable by day 13 (Theiler, 1972).

1.3. EMBRYONAL CARCINOMA CELLS

1.3.1. Summary

Embryonal carcinoma (EC) cells are the stem cells of teratocarcinomas. The latter are solid tumours, usually of the gonads, which contain a variety of differentiated cell types in addition to the malignant stem cells (reviewed by Stevens, 1983). EC cells are analogous to early embryonic cells in that they are "pluripotential", that is they can differentiate into a wide range of cell types. Teratocarcinomas in general contain derivatives of all three embryonic germ

layers: ectoderm (neural tissue, for example), mesoderm (such as muscle and cartilage), and endoderm (e.g. glandular structures). Extra-embryonic tissues, notably yolk sac, are also commonly present. The undifferentiated stem cells are randomly distributed throughout the tumour in small clusters.

Malignancy is associated with the presence of EC cells. When they are absent, due to differentiation and/or necrosis, the tumours are benign and known as teratomas (though this is also employed as a collective term for both benign and malignant tumours). The presence of EC cells also confers serial transplantability on the tumours in some cases.

The undifferentiated cells can be isolated from teratocarcinomas or from their ascitic forms (known as embryoid bodies, see following section) and propagated *in vitro*. Several clonal EC lines are now available. They give rise to teratocarcinomas when injected back into mice. EC cells differentiate in culture in response to a variety of stimuli. This facilitates their use for the study of determination, the process by which pluripotent cells become committed to a particular developmental pathway, and of differentiation *per se* (Martin, 1975; 1980). As *in vivo*, differentiation *in vitro* is accompanied by a loss of malignancy.

EC cells resemble early embryonic cells (inner cell mass/primitive ectoderm) morphologically, ultastructurally, biochemically, antigenically and in their developmental potential (reviewed by Martin, 1980). When aggregated with host ICMs or micro-injected into host blastocysts, they may participate in normal development, generating chimaeric progeny with EC-derived contributions to many tissues and cell types (reviewed by Papaioannou and Rossant, 1983). This has led to the suggestion that EC cells are normal embryonic stem cells which are malignant only when they occur outside of the blastocyst environment. Recently strong evidence in support of this idea has come from the isolation of EC-like cells directly from implantation stage mouse embryos (Evans and Kaufman, 1981; Martin, 1981; Axelrod, 1984). These embryonic stem cell lines are called EK cells and share many properties with teratocarcinoma-derived EC lines.

1.3.2. Origins of Teratomas

Spontaneously occurring teratomas are very rare in nature. They arise at relatively high frequency in certain inbred strains of mice, however. This was first discovered for strain 129 mice by Stevens and Little (1954). Approximately 1% of males of this strain have spontaneous testicular teratomas. These become apparent in the foetal genital ridge from 15 days of gestation and their initial development occurs within the seminiferous tubules. They are presumed to arise from primordial germ cells (Stevens, 1983), which bear a close morphological and ultrastructural resemblance to EC cells (Pierce and Beals, 1964) and are likewise pluripotent.

Teratomas can also be induced experimentally in several mouse strains by grafting genital ridges from 12.5 day male foetuses to adult testes (reviewed by Stevens, 1983). Around 80% of such grafts result in teratomas in 129 mice. Genetic evidence in support of the germ cell origin of such tumours is provided by the finding (Stevens, 1967) that teratomas can only rarely be obtained from 129 mice carrying a congenital deficiency of germ cells (homozygous for the *Sl* allele).

Spontaneous ovarian teratomas occur in about half of strain LT/Sv female mice (Stevens and Varnum, 1974). These originate from eggs which begin to develop parthenogenetically within the ovary. Such parthenogenetic embryos develop to early egg cylinder stages, then become disorganised and teratomatous. Ovarian teratomas differ from testicular teratomas in that they usually contain trophoblastic giant cells. Stevens (1983) considers that this may be a consequence of their development via blastocysts, which possess a trophectoderm component.

Teratomas can be generated at high frequency by transplantation of 1–7 day embryos to ectopic sites provided the foetal component of the embryo is included in the graft (Solter and Damjanov, 1973; Stevens, 1983). Both male and female embryos can give rise to teratomas in many mouse strains using this technique. In such cases teratomas appear to develop directly from disorganised populations of pluripotent embryonic cells, in contrast to testicular and ovarian teratomas which develop from primordial germ cells (Stevens, 1983).

Most testicular, ovarian and embryo-derived teratomas are benign. Even those which contain undifferentiated cells often cease growing when transplanted. Occasionally, however, teratocarcinomas continue growing after transplantation. Transplantable teratocarcinomas, however derived, are similar in composition during early transplant generations, containing a wide variety of cells and tissues. With successive retransplantation the EC cells usually become progressively restricted in developmental potency so that tumours contain only a few or just a single differentiated cell type (Stevens, 1983). Eventually the teratomas may become benign due to loss of all the stem cells.

Some transplantable teratocarcinomas grow as ascitic tumours when injected intraperitoneally (Pierce and Dixon, 1959;). They develop into multiple small aggregates which resemble early embryos and are therefore termed embryoid bodies. These structures float freely in the ascitic fluid. Simple embryoid bodies consist of EC cells surrounded by an envelope of endoderm. The so-called cystic embryoid bodies are more complex and resemble slightly later (Day 6 onwards) stages of embryonic development. They contain a fluid-filled cyst in addition to EC cells and/or a variety of differentiated tissues.

1.3.3. Isolation of EC Cells and their Propagation *In Vitro*

EC cells can be isolated from solid transplantable teratocarcinomas (Evans, 1972; Martin and Evans, 1974,1975a; Jami and Ritz,1974) or, more readily, from embryoid bodies (Finch and Ephrussi, 1967; Kahan and Ephrussi, 1970; Rosenthal *et al*, 1970; Jakob *et al*, 1973; Bernstine *et al*, 1973). Dissociated pieces of tumour or embryoid bodies (usually left intact) are placed in culture, either alone or in the presence of a feeder layer (Kahan and Ephrussi, 1970; Martin and Evans, 1975a). Clonal EC lines can subsequently be derived from the mixed population primary (or secondary) cultures. These clonal lines may produce homogeneous EC cell cultures or heterogeneous cultures containing varying proportions of EC cells. For obvious reasons uniform EC populations are more useful for analysis of determination and differentiation than heterogeneous cultures.

Maintenance of the stem cell population *in vitro* is often dependent on the presence of a feeder layer of non-dividing mouse fibroblasts (Martin and Evans, 1975a) in which case the cell line is described as feeder-dependent.

Feeder-independent cell lines, in contrast, can be propagated directly on tissue culture plastic with little or no spontaneous differentiation. Coating the substratum with gelatin often facilitates the culture of feeder-independent EC cells, presumably via effects on cell attachment (Bernstine *et al*, 1973). It is also possible to maintain relatively pure EC populations from some spontaneously differentiating lines without feeders by adhering to a strict subculturing regime (Lo and Gilula, 1980a).

EC cells multiply rapidly in culture, with doubling times of 8-16 hours (Rosenstraus *et al*, 1982; Sennerstam and Stromberg, 1984). They are not subject to density-dependent inhibition of growth, though in some lines differentiation is promoted at high density.

EC cells are exceptional amongst established cell lines in possessing near-normal karyotypes. Many are apparently euploid but high resolution karyological analyses indicate that most, if not all, teratocarcinoma cells maintained *in vitro* bear some chromosomal abnormalities (reviewed by Solter and Damjanov, 1979). There is some evidence of an association between deviation from euploidy and loss of differentiation capacity (Iles and Evans, 1977).

1.3.4. Characterisation of EC Cells

In vitro EC cells grow as tightly packed epithelioid colonies. The cells are small (12-14μm in diameter) with a high nucleus to cytoplasm ratio and prominent nucleoli. At the ultrastructural level the cytoplasm contains few mitochondria, small amounts of endoplasmic reticulum, primitive Golgi, and large numbers of free ribosomes (Martin, 1975; Lo and Gilula, 1980b). They resemble embryonic cells of the ICM and primitive ectoderm (Wiley *et al*, 1978; Enders *et al*, 1978) and also primordial germ cells (Pierce and Beals, 1964).

As yet no exclusive biochemical markers of embryonal carcinoma have been identified. EC cells possess high alkaline phosphatase activity (Bernstine *et al*, 1973) in common with ICM and embryonic ectoderm cells. This can be used to distinguish EC cells histochemically from their differentiated derivatives which have significantly lower levels of this enzyme (Bernstine *et al*, 1973; Koopman and Cotton, 1984). Conversely, EC cells do not express specific differentiated functions, such as production of plasminogen activator or AFP, for example,

which are markers of parietal and visceral endoderm respectively (Section 1.2.2.).

EC cells do not produce an obvious extracellular matrix, yet they do synthesize certain extracellular matrix components, including type IV collagen (or some closely related species) (Adamson *et al*, 1979) and fibronectin (Wolfe *et al*, 1979). Laminin and entactin are made only in trace amounts, however (Kurkinen *et al*, 1983). EC cells lack keratins and exhibit little cytoskeletal organisation (Nicolas *et al*, 1981a).

EC cells are permissive for certain viruses but not others, including polyoma and SV40 (reviewed by Nicolas *et al*, 1981a). The latter undergo chromosomal integration but gene expression is repressed (Swartzendruber *et al*, 1977). On induction of differentiation, however, the cells become permissive for polyoma growth and SV40 T antigen expression (Swartzendruber and Lehman, 1975; Boccara and Kelly, 1978). This property can be used to monitor differentition and to investigate mechanisms of regulation of gene expression (Tanaka *et al*, 1983; Taketo *et al*, 1983).

The protein synthesis patterns of pluripotential EC lines as revealed by SDS disc gel electrophoresis (Martin *et al*, 1978) and by two dimensional gel electrophoresis (Dewey *et al*, 1978; Lovell-Badge and Evans, 1980) differs markedly from that exhited by ICM cells. Using the same approach Evans *et al* (1979) found that PSMB EC cells show homology with epiblast cells of the early post-implantation embryo (5.5 days post-coitum, see Section 1.2.). but differ from later ectodermal cells and are clearly distinct from primordial germ cells. These workers also demonstrate that Forssman antigen, which is expressed by EC cells and implantation stage embryonic cell masses (and endoderm), disappears from ectodermal cells during differentiation of the egg cylinder (6.5-7.5 days post-coitum). These biochemical and antigenic indications of the equivalence of EC cells and primitive ectoderm of the 5.5 day embryo are borne out by the developmental potentials of many EC cells *in vivo* (Section 1.3.5.; but see also Section 1.4.).

Cell surface antigens provide useful markers for undifferentiated embryonal carcinoma. Antigens such as SSEA-1 (Solter and Knowles, 1978) are lost as EC cells differentiate and monoclonal antibodies against these determinants can therefore be used to monitor the differentiation process. EC cells have been

widely employed to generate antisera and monoclonal antibodies against cell surface molecules which may serve as lineage markers and/or play important roles in early embryonic development. Such antibodies generally cross-react with pre-and early post-implantation stage mouse embryos but are unreactive against many or most terminally differentiated adult cell types (Silver *et al*, 1983a). Products of the major histocompatibility locus, H2, cannot be detected on EC cells, which further distinguishes them from most differentiated cell types (Nicolas *et al*, 1981a).

Antibodies raised against embryonal carcinoma do not react uniformly with all EC cells (Nicolas *et al*, 1981a) and in certain cases reactivity is restricted to specific lines (Nicolas *et al*, 1981b; Silver *et al*, 1983a). Variability in cell surface composition is only one of several examples of heterogeneity between different EC isolates (Nicolas *et al*, 1981a; Gardner, 1983b). Others include junctional communication properties (Nicolas *et al*, 1981a), whether one or both X chromosomes are active in female lines (McBurney and Adamson, 1976; Martin *et al*, 1978), dependence on feeders (Section 1.3.2.), developmental potential *in vivo* (Section 1.3.4.), differentiation behaviour *in vitro* (Section 1.4.) and response to chemical inducing agents (Section 1.5.). This heterogeneity is primarily uncontrolled, presumably having its origins in both the diverse sources and isolation procedures of these cells (Silver *et al*, 1983b) and in cell selection in abnormal environments both *in vivo* and *in vitro* (Gardner, 1983b). It constitutes the first major disadvantage of the application of EC cells to the study of embryogenesis It implies that different EC cell lines may differ in their developmental status and/or in the extent of their deviation from normal embryo cells, and renders difficult comparisons between studies on different cell lines. The second major limitation of the use of EC cells as models of normal development is that the proximate cause of teratocarcinogenesis is completely unknown (Gardner, 1983b). Consequently EC cell lines cannot be derived under defined conditions and their precise relation to normal embryonic cells remains obscure.

The isolation of pluripotent cells directly from embryos (Section 1.6.) overcomes both of these problems and therefore represents a considerable advance in mammalian embryology.

1.3.5. Developmental Potential of Embryonal Carcinoma Cells

It has been clearly established that most clonal EC lines remain pluripotent, since they give rise to multi-differentiated teratocarcinomas when injected into syngeneic mice. Subclones also generate differentiated tumours, demonstrating that the pluripotency of individual cells is maintained during growth *in vitro*. Production of teratocarcinomas by subcutaneous injection generally requires inoculation of 10^4–10^6 EC cells. Rayner and Graham (1982) have investigated tumourigenicity in the generations immediately after cloning by grafting small clonal populations under the kidney capsules of syngeneic mice. Under these conditions, teratocarcinomas developed from 33% of cell pairs derived from a single EC cell.

The developmental potency of EC lines does vary, however. Some differentiate poorly in tumours while others produce well-differentiated tumours containing derivatives of all three germ layers. In general, feeder-dependent lines have greater developmental potency than feeder-independent lines (Martin and Evans, 1975a,b; Hogan, 1976; Nicolas *et al*, 1981). This may reflect a genuine difference in the developmental stage of the EC cells or may be due to some genetic or epigenetic adaptation to culture conditions. Emancipation from feeders is not incompatible with the capacity for multiple differentiation *in vivo*, however (Stewart and Mintz, 1981). Pluripotency also tends to become gradually reduced with increasing time in culture. Some lines have completely lost the ability to differentiate *in vivo* (Bernstine *et al*, 1973; Martin and Evans, 1975a) and are described as nullipotent. Nullipotent EC cells give rise to tumours consisting solely of embryonal carcinoma cells. They do not differentiate spontaneously *in vitro*, but may respond to chemical inducers (Section 1.5.).

EC cells may also differentiate in chimaeric embryos produced by blastocyst injection or via aggregation of the teratocarcinoma cells with morulae (Brinster, 1974; Papaioannou *et al*, 1975; Mintz and Illmensee, 1975; Fujii and Martin, 1983). The EC cells may contribute to both extraembryonic and foetal tissues and in many cases the embryos develop normally to term and generate chimaeric adult mice (reviewed by Papaioannou and Rossant, 1983). There appears to be no tissue restriction within the foetal soma. Moreover, two lines maintained as transplantable tumours and one euploid *in vitro* cell line have

been shown to colonise the germ line in chimaeras and give rise to functional EC-derived gametes, thus demonstrating that EC cells are genetically totipotent (Mintz *et al*, 1975; Cronmiller and Mintz, 1978; Stewart and Mintz, 1981). This creates the possibility of employing EC cells as vehicles for the introduction of selected genotypes and foreign genes into mice (reviewed by Stewart, 1985).

The participation of EC cells in normal embryogenesis following their introduction into the blastocyst provides compelling evidence of their similarity to pluripotent embryonic stem cells and thus validates their use as *in vitro* models of mouse development. This apparent normalisation of the malignant phenotype by the embryonic environment has also stimulated research into EC cells by tumour biologists interested in the relationship between stem cell proliferation and differentiation (Pierce *et al*, 1983; Gardner, 1983b). However, there are differences between EC-embryo and embryo-embryo chimaeras (reviewed by Papaioannou and Rossant, 1983). Not only do EC cells contribute less often than ICM cells to offspring, but the contributions are often small and sporadic with germ line mosaicism being very rare. Furthermore, EC chimaeras develop tumours, generally teratocarcinomas, with relatively high frequency and in some cases exhibit developmental abnormalities (Rossant and McBurney, 1983). These phenomena vary with the EC line under investigation but no correlations have yet been established with *in vitro* characteristics, excepting of course the requirement of euploidy for generation of gametes. This variability has limited the application of EC chimaeras as developmental probes and as potential means of genetic manipulation. The isolation of stem cells directly from embryos (Section 1.6.) promises to circumvent these difficulties, however, as such EK cells contribute to chimaeras at high frequency, including in some cases colonisation of the germ line (Bradley *et al*, 1984).

The finding that some EC cell lines respond normally to developmental cues after blastocyst injection, whilst others do not, suggests that different teratocarcinoma stem cells may differ in their relationship to embryonic stem cells. Specifically, some EC cell lines may be closely analogous to embryonic stem cells, whereas others may represent deviations selected, during growth in tumours or in culture, for continuous proliferation and non-responsiveness to normal developmental signals. In this instance, however, it may prove possible to employ the heterogeneity of EC cell lines constructively in efforts to identify developmental signals via analyses of chimaera development *in vitro* (Rossant and McBurney, 1983; Papaioannou and Rossant, 1983).

1.4. DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS IN VITRO

EC cells differentiate under a variety of conditions *in vitro*. The stimulus may be provided by the culture conditions, by the three dimensional arrangement of the cells, by biosynthetic macromolecules, or by chemical inducing agents.

In certain feeder-independent EC lines plating at low density may be followed by a high level of spontaneous differentiation (Burke *et al*, 1978). In others differentiation is promoted by culture at high density (Evans and Martin, 1975; Lo and Gilula, 1980a; Nicolas *et al*, 1981a). Some multipotent EC cells which exhibit low levels of spontaneous differentiation may be triggered to differentiate by culture in defined serum-free medium (Darmon *et al*, 1982; Rizzino,1983a). This may be due to the presence in such media of either laminin or fibronectin since differentiation of the same EC cell lines is promoted in serum-containing medium by pre-coating culture dishes with either of these macromolecules (Rizzino, 1983b). Low concentrations of exogenous laminin have also been reported to stimulate endoderm maturation in embryoid bodies (see below) formed from the EC line F9 (Adamson and Grover, 1983). These observations suggest that certain extracellular matrix components may play a direct role in the control of cellular differentiation, particularly in the formation of the extraembryonic endoderm. Medium containing delipidized serum has also been reported to promote differentiation of EC cells (Sherman *et al*, 1983a). It therefore appears that complete serum contains a factor or factors which prevent differentiation and over-ride the effects of "natural" inducers.

Feeder-dependent teratocarcinoma cell lines were originally reported to die on monolayer culture in the absence of a feeder layer (Martin and Evans, 1975a). Subsequently it has been found that cell viability can be maintained by the presence of 2-mercaptoethanol (Oshima,1978; Chapter 7 below) and under these conditions feeder-dependent EC cells differentiate spontaneously in monolayer culture. Such differentiation is reduced by feeder-conditioned medium (Smith and Hooper, 1983; Koopman and Cotton, 1984), implying that feeders produce a diffusible factor or factors which inhibit differentiation and/or promote stem cell differentiation..

Both feeder-dependent and feeder-independent EC lines may be triggered to differentiate via the formation of cell aggregates. Aggregation can be induced

in several ways (reviewed by Martin and Evans, 1975a). Feeder-dependent cells may be plated at high density in the absence of feeders and 2-mercaptoethanol, in which case rounded clumps of viable cells form. These clumps become detached from the substratum either spontaneously or following mechanical agitation. Alternatively cells can be seeded at low density on feeders, then individual colonies transferred intact to vessels with a non-adhesive substratum (bacteriological dishes, for example). Aggregates can also be obtained by passaging poorly dissociated cultures directly into bacteriological dishes.

Within 12-36 hours of detachment of certain pluripotent EC cells, such as PSA4 and its derivatives used in this study, an outer layer of endodermal cells becomes apparent on the surface of the aggregates. These structures bear a close resemblance to the simple embryoid bodies formed by ascitic teratocarcinomas *in vivo* (Section 1.3.2.), based on ultrastructural analysis and histochemical stains for Reichert's membrane and for alkaline phosphatase (Martin and Evans, 1975a,b). The former is produced by endodermal cells and the latter is found at high levels in EC cells (Bernstine *et al*, 1973; see Section 1.3.4.). Subsequently the endodermal phenotype of the outer cells has been confirmed by their production of plasminogen activator and expression of markers such as laminin, AFP and transferrin (see Section 1.2.2.) (Hogan *et al*, 1981; Adamson, 1982; Grabel *et al*, 1983; Kurkinen *et al*, 1983). Nullipotent EC cells by contrast form homogeneous aggregates of embryonal carcinoma *in vitro* as *in vivo*.

In some lines, including PSA4, embryoid bodies rapidly become complex and cystic, whilst in others there is no further development so long as they are maintained in suspension (Martin and Evans, 1975a,b; Martin *et al*, 1977). When allowed to reattach to the substratum, however, both types of embryoid body give rise to outgrowths containing a variety of differentiated cell types, including derivatives of all three foetal germ layers (Martin and Evans, 1975c).

The formation of simple embryoid bodies resembles the delamination of endoderm on the free surface of the ICM of the implantation stage mouse embryo. Isolated ICMs have been found to form endoderm over the whole of their outer surfaces (Rossant, 1975; Gardner and Papaioannou, 1975). However, primitive ectoderm cores isolated from implantation stage blastocysts do not regenerate endoderm (reviewed by Gardner, 1983a). Thus the capacity

exhibited by certain EC lines to differentiate into extraembryonic endoderm on aggregation suggests that they are developmentally equivalent to ICM cells, apparently in conflict with the biochemical evidence provided by protein synthesis profiles (Section 1.3.4.). This discrepancy has not been satisfactorily resolved (Martin, 1980). It is possible that EC cells may possess greater developmental lability than normal primitive ectoderm cells by virtue of their transformation and/or selection *in vivo*. However, embryonic stem cells (EK cells, Section 1.6.), isolated directly from the epiblasts of implantation stage embryos, can also form embryoid bodies and regenerate endoderm (Doetschmann *et al*, 1985). Selection for continuous growth *in vitro* may introduce developmental reversion in this instance. Alternatively, the development of intact primitive ectoderm cores may be regulated by epigenetic factors rather than genetically restricted (Gardner, 1983).

The transition from simple to cystic embryoid body shares many common features with the development of isolated ICMs *in vitro* (Martin *et al*, 1977; Wiley *et al*, 1978) and is analogous in some respects to differentiation of the egg cylinder in the early post-implantation embryo (Section 1.2.2.). Thus between the fourth and sixth day of suspension culture one or occasionally more cavities appear and the surrounding EC cells elongate to form a simple columnar epithelium of cells connected by specialized intercellular junctions and resembling mature embryonic ectoderm (Section 1.2.2.). The cavities generally form eccentrically and are associated with focal necrosis (Martin *et al*, 1977; Boyd *et al*, 1984). Similar cavities appear in cultured ICMs, though in this case a large proportion develop into paired cysts (Wiley *et al*, 1978). Cavitation of embryoid bodies shows some similarities to the process of proamniotic cavity formation in the egg cylinder, though there are differences in detail (Boyd *et al*, 1984). Embryoid bodies which do not cavitate undergo no further differentiation whilst maintained in suspension (Martin *et al*, 1977; Uno, 1982). Evans and Martin (1975) have proposed that this may be because the cavity provides a two-dimensional surface for the positional organization necessary for further cell determination. This would account for the differentiation of such non-cavitating embryoid bodies which proceeds if they are allowed to reattach to a substratum (Evans and Martin, 1975; Martin and Evans, 1975a).

Cavitated embryoid bodies in suspension culture often develop large fluid-filled cysts, parallelling the formation of visceral yolk sac in utero (Section 1.2.2.). These cysts may be lined with mesodermal derivatives, including capillaries,

blood islands and cardiac muscle (Martin *et al*, 1977; Uno, 1982). Various differentiated cell types may also be present in the core of the embryoid body, which becomes increasingly disorganised (Martin and Evans, 1975c; Martin *et al*, 1977). Mesoderm formation in embryoid bodies in general appears to occur via a different pathway from that obtaining in the egg cylinder, however. In the latter, presumptive mesoderm cells delaminate from the embryonic ectoderm during the morphogenetic process of primitive streak formation and migration to line the yolk sac results in formation of the extraembryonic mesoderm (Section 1.2.2.). The trilaminar arrangement of ectoderm, mesoderm and endoderm is rarely seen in embryoid bodies. Instead it appears that mesoderm differentiation proceeds directly from the attenuated ectodermal layer produced by expansion of the cavity (Martin *et al*, 1977). Similar developments occur in cultured ICMs (Wiley *et al*, 1978) indicating that they arise as a consequence of the *in vitro* environment rather than from some abnormality of the EC cells.

Another difference between the development of embryoid bodies and that of the egg cylinder is that whereas epiblast cells in utero become invested exclusively with visceral endoderm (Section 1.2.2.), embryoid bodies always contain a high proportion of parietal endoderm (Martin *et al*, 1977). Isolated ICMs may also give rise to parietal endoderm under appropriate conditions, however (Strickland *et al*, 1976; Gardner, 1983). As differentiation into extraembryonic endoderm is believed to proceed via bipotential primitive endoderm (Section 1.2.), it is perhaps not surprising that both mature phenotypes can be obtained. Indeed Hogan and colleagues (Hogan *et al*, 1981, 1983; Kurkinen *et al*, 1983) have shown that it is possible to modulate production of either visceral or parietal endoderm from aggregates of the EC line F9 (see following Section).

The EC line S2 forms only simple embryoid bodies (Martin and Evans, 1975a,c) and these exhibit little if any visceral endoderm (Martin *et al*, 1977). Uno has also reported that non-cavitating embryoid bodies apparently lack visceral endoderm. This has led to the proposal (Martin *et al*, 1977) that visceral endoderm may be required for development of embryoid bodies beyond the two-layered stage, presumably via the provision of some inductive signal. Rosenstraus *et al* (1983) have reported that cavitation does occur in mixed aggregates of EC and parietal endoderm cells. However, these workers demonstrate neither the mosaic nature of the embryoid bodies in question, nor the absence of visceral endoderm.

Both cavitating and non-cavitating embryoid bodies give rise to a variety of tissues and cell types when allowed to attach to a suitable growth surface, though cystic structures generally generate a greater spectrum of differentiation (Evans and Martin, 1975; Martin and Evans, 1975c). The differentiation capacity displayed by EC lines in such embryoid body outgrowths parallels their developmental potential in tumours (Martin and Evans, 1975c).

Most EC lines do not delaminate endoderm following aggregation, however. In some, aggregation induces commitment such that the cells differentiate when replated onto culture surfaces (Nicolas *et al*, 1981a). In others no differentiation occurs during or after aggregation. Thus, as with all the other stimuli for differentiation discussed in this Section, not only do different EC lines give rise to different products in different ways, but many fail to respond at all. This illustrates the heterogeneity amongst EC cell lines discussed in Section 1.3.4.

1.5. INDUCTION OF EMBRYONAL CARCINOMA CELL DIFFERENTIATION BY CHEMICALS

1.5.1. Retinoic Acid and Other Chemicals Which Induce Embryonal Carcinoma Differentiation

Various chemicals have been shown to promote the differentiation of EC cells *in vitro*. Notable amongst these are vitamin A and related compounds, the retinoids (Fig.1.3). The acid derivative, retinoic acid, is the most effective, with the all-*trans* isomer being slightly more potent than the 13-*cis* form. This is the only agent yet found which stimulates differentiation of all EC lines tested (with the exception of a few specifically selected variants, see following Section), including several which otherwise appear nullipotential *in vitro* and *in vivo* (Strickland and Mahdavi, 1978; Jetten *et al*, 1979; Adamson *et al*, 1979; Jones-Villeneuve *et al*, 1982; Matthaei *et al*, 1983).

Although retinoic acid exhibits marked toxic effects in some instances (Strickland and Mahdavi, 1978; Jetten *et al*, 1979), its effects on differentiation are inductive rather than selective. Thus it is effective at clonal density on EC lines which harbour few, if any, differentiated derivatives during normal culture

(Strickland and Mahdavi, 1978; Jetten *et al*, 1979). Rayner and Graham (1982) confirmed this conclusion by an elegant analysis of the change in growth phenotype following exposure to retinoic acid.

Retinoic acid can induce transient changes in morphology, growth behaviour, cell surface properties, protein expression and membrane lipid composition in several cell types, which are manifest only in the continuous presence of the retinoid (Jetten *et al*, 1979; Lotan, 1980; Ringler *et al*, 1984). In EC cells, by contrast, a true differentiation response is engendered whereby the new phenotype is stable in the absence of retinoic acid and is transmitted to subsequent generations (Strickland and Mahdavi, 1978). Moreover, retinoic acid can be removed prior to overt cytodifferentiation (Rayner and Graham, 1982; Jones-Villeneuve *et al*, 1982), indicating that it acts on the process of cell commitment rather than on differentiation *per se* (see below and following Section for further discussion).

The differentiated nature of the cells obtained following retinoid treatment has been established by a variety of methods depending on the particular cell line. The feeder-independent PC13.5 line (Hooper and Slack, 1977) employed in this study gives rise predominantly to flattened, epithelioid derivatives referred to as END cells (Adamson *et al*, 1979). These are morphologically distinct from PC13.5 EC cells and differ in their growth behaviour, having a longer generation time and a finite proliferative lifespan (Rayner and Graham, 1982; Mummery *et al*, 1984; Rayner and Pulsford, 1985). PC13.5 END cells are non-tumourigenic (Rayner and Graham, 1982; see below) and have lost the capacity for growth in semi-solid media (Heath, 1983). They express cell surface receptors for and are mitogenically responsive to epidermal growth factor (EGF) and insulin, in contrast to their undifferentiated progenitors (Rees *et al*, 1979; Heath *et al*, 1981). The differentiated cells lack SSEA-1 and secrete plasminogen activator (Muummery *et al*, 1984). They show enhanced production of type I collagen (Adamson *et al*, 1979) but continue to express type IV procollagen and do not produce transferrin (Adamson, 1982). Thus they do not correspond closely to either parietal or visceral endoderm (Section 1.2.2.). Their precise identity remains uncertain but they are now believed to represent mesodermal derivatives on the basis of cell surface antigen expression (J. Heath, personal communication).

The nature of the differentiated cell type(s) produced in response to retinoic

acid depends on the particular EC line under investigation (Jetten *et al*, 1979). In general, derivatives obtained after retinoid treatment are similar to those which arise spontaneously (often at very low frequency) during normal culture of the same cell line. Thus retinoic acid does not determine the form of differentiation but rather promotes the developmental direction preferred by the EC line in question. This can be modulated by the spatial organization of the cells and by interaction with other chemicals.

The clearest example of this is provided by the response of F9 EC cells (reviewed by Hogan *et al*, 1983). F9 exhibits a very low level of spontaneous differntiation into endoderm, both in monolayer culture and in aggregates, which is greatly enhanced by treatment with retinoic acid (Strickland and Mahdavi, 1978; Jetten *et al*, 1979). Either parietal or visceral endoderm cells can be obtained depending on the culture conditions. In monolayer culture parietal endoderm-like cells are generated which produce tissue plasminogen activator (Strickland and Mahdavi, 1978), laminin and type IV procollagen (Strickland *et al*, 1980), and do not synthesize AFP (Strickland and Sawey, 1980). This response is greatly increased by the presence of cyclic AMP, or agents which elevate intracellular cyclic AMP, during or following exposure of the F9 cells to retinoic acid. Additional morphological changes occur under such conditions, the cells becoming rounded with numerous filipodia. These cells exhibit many features of mature parietal endoderm (Hogan *et al*, 1983). Retinoic acid treatment of floating aggregates of F9 stimulates the formation of a continuous outer layer of endodermal cells (Hogan *et al*, 1981). These cells display the ultrastructural properties of a functioning epithelium of visceral endoderm; polarized cytoplasm, numerous apical microvilli, subapical vacuoles and endocytic vesicles, apical junctional complexes, and a basement membrane (Hogan *et al*, 1983; Adamson and Grover, 1983; see Section 1.2.2.). Such aggregates produce the visceral endoderm marker AFP which is localised to the outer layer by immunoperoxidase and immunofluorescence tests (Hogan *et al*, 1981; Adamson and Grover, 1983). In the presence of cyclic AMP, however, mature visceral endoderm is not formed. Instead the cells develop the phenotype of parietal endoderm, producing large amounts of extracellular matrix and failing to express AFP (Hogan *et al*, 1981, 1983).

Hogan and co-workers (1981, 1983) propose that these observations are consistent with an initial differentiation of F9 into bipotential primitive endoderm cells. These can express their capacity for epithelial organisation if

they remain in contact with other cells. In the absence of intercellular contacts with an adjacent cell layer, or in the presence of high cyclic AMP, however, they develop the mature parietal phenotype. This hypothesis is supported by the finding that high density monolayer cultures of F9, in which the cells become multilayered, give rise to cells of both the visceral and parietal phenotypes when treated with retinoic acid (Hogan *et al*, 1981).

The differentiation of F9 *in vitro* shares many common features with the development of extraembryonic endoderm in the early mouse embryo (Section 1.2.). This relationship is summarised in Fig.1.2.

It has also been reported that neural differentiation of F9 can be induced by monolayer culture in the presence of retinoic acid and dibutyryl cyclic AMP for several days (Kuff and Fewell, 1980), an effect potentiated by nerve growth factor (Liesi *et al*, 1983; see Section 1.7.). It is not clear how this is related to the preceding observations on endodermal differentiation. F9 was originally multipotent (Bernstine *et al*, 1973) and it is known that considerable biological variation may occur between different sub-lines (Adamson and Grover, 1983). In some cases subpopulations capable of neural differentiation are evidently still present. Whether the same cells can also develop into endoderm is unresolved.

The interaction of retinoic acid with the pluripotent EC line P19 and its derivatives (McBurney *et al*, 1983) is even more complex. In monolayer cultures retinoic acid induces differentiation into fibroblasts (Jones-Villeneuve *et al*, 1982, 1983). However, if these cells are exposed to retinoic acid whilst aggregated a variety of differentiated cell types may be generated, which grow out on reattachment to a culture surface. The particular form of differentiation adopted is dependent on the concentration of retinoid used (Edwards and McBurney, 1983). McBurney and colleagues (McBurney *et al*, 1983; Edwards and McBurney, 1983) have therefore suggested that concentration gradients of retinoic acid (or some agent whose effects are mimicked by retinoic acid) may play an important morphogenetic role in embryonic development. Interestingly, retinoids have been shown to perturb pattern formation in developing and regenerating limbs (Tickle *et al*, 1982; Summerbell, 1983; Maden, 1982).

The response of feeder-dependent EC cells to retinoic acid remains unclear because of the difficulty of unambiguously distinguishing differentiated EC derivatives from feeder cells. Moreover, feeders inhibit the induction of

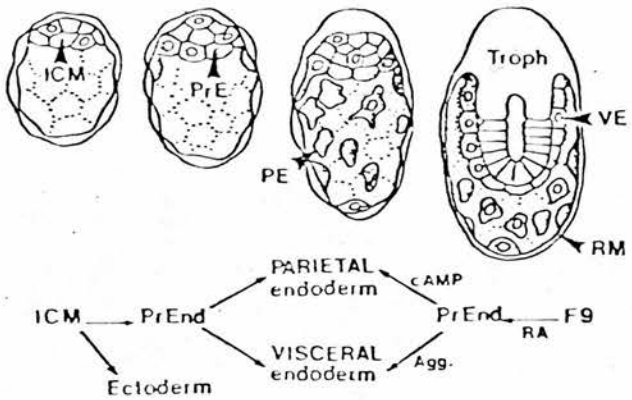
Fig.1.2 Differentiation of Extraembryonic Endoderm in The Mouse Embryo and F9 EC Cells

From Kurkinen *et al* (1983)

Fig.1.3 The Basic Structural Formula of Retinoids

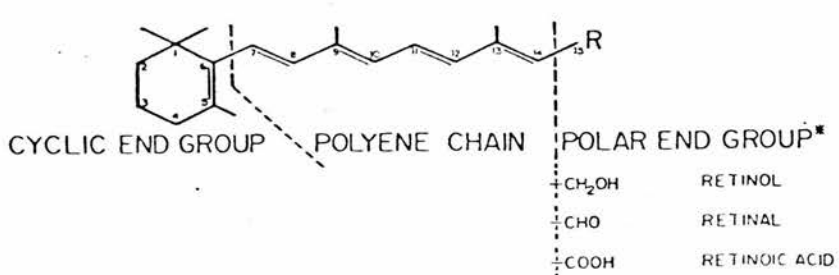
Adapted from Lotan (1980)

FIG 1.2 Differentiation of Extraembryonic Endoderm in the Mouse Embryo and F9 EC Cells



Differentiation of parietal and visceral endoderm in the mouse embryo and F9 teratocarcinoma cells. Primitive endoderm (PrEnd) cells in the fifth-day blastocyst generate both parietal and visceral endoderm (PE) and (VE). Individual PE cells migrate over the trophoblast (Troph) and lay down Reichert's membrane (RM), whereas VE cells form a continuous epithelium around the growing egg cylinder. It is thought that RA induces F9 EC to differentiate into PrEnd. cAMP then increases the probability of differentiation into PE, whereas close contact with EC cells and/or conditions allowing the assumption of a polarized, epithelial morphology promotes differentiation into VE (Hogan et al. 1981).

FIG 1.3 Basic Structural Formula of Retinoids



differentiation of feeder-independent EC cells by retinoic acid (Ogiso *et al*, 1982; Rosenstraus *et al*, 1984). This effect is mediated, at least in part, via diffusible factors since it is reproduced by medium conditioned by incubation with feeder cells (Smith and Hooper, 1983).

Differentiation of EC cells following exposure to retinoic acid is accompanied by a loss of tumourigenicity (Sherman *et al*, 1981; Rayner and Graham, 1982). Strickland and Sawey (1980) have shown that the growth of F9-derived teratocarcinomas *in vivo* is reduced and differentiation increased in mice whose diet is supplemented with retinoic acid. This has heightened interest in the possible therapeutic application of retinoids as anti-tumour agents (reviewed by Sporn and Roberts, 1983).

Various compounds structurally unrelated to retinoids can promote differentiation of EC cells, though these are restricted in action to a few, or even to a single EC line. Many of these substances are inducers of Friend murine erythroleukaemia cell differentiation, as indeed is retinoic acid (Garg and Brown, 1983). They include hexamethylenebisacetamide (HMBA) (Speers *et al*, 1979; Nicolas *et al*, 1981a), N',N-dimethylacetamide and polybrene (Speers *et al*, 1979), 5-bromodeoxyuridine (Speers and Lehman, 1976), butyrate (McBurney *et al*, 1982; McCue *et al*, 1984a), 6-thioguanine and DMSO (McBurney *et al*, 1982; Edwards *et al*, 1983). In some cases their action is dependent on or modulated by cellular interactions (Speers *et al*, 1979; McBurney *et al*, 1982; Campione-Piccardo *et al*, 1985b). Other agents reported to induce EC differentiation include the inhibitors of poly(ADP-ribose) synthetase, 3-aminobenzamide and nicotinamide (Ohashi *et al*, 1984), and 2-difluoromethylornithine (Schindler *et al*, 1983; Jetten and Shirley, 1985), an irreversible inhibitor of ornithine decarboxylase.

1.5.2. The Mode of Action of Inducing Agents

One of the principal aims of research into embryonal carcinoma cells has been to elucidate the underlying biochemistry of commitment, the process by which pluripotent stem cells make the decision to differentiate. The advent of chemical inducers, particularly the universally acting retinoic acid, appeared to make this prospect a reality. Only limited progress has yet been made, however, and several conflicting theories persist on the mode of action of

retinoids. Moreover, although retinoic acid is a naturally occurring substance (plasma concentration 10^{-8}–10^{-9}M, Alam and Alam, 1983), the relevance of chemically induced differentiation *in vitro* to events *in vivo* remains uncertain.

As discussed in the previous Section, it appears that the effect of retinoic acid is to induce a decision to differentiate, but that the developmental outcome of this decision is determined by genetic and environmental factors. Even in the case of P19 EC cells, which form different cell types in response to different concentrations of retinoic acid (see previous Section), the retinoid can be removed prior to the aggregation step which is essential for generation of diverse cell types (Jones-Villeneuve *et al*, 1982). The proportion of committed cells in the aggregates, which is dependent on the concentration of retinoic acid employed (Campione-Piccardo *et al*, 1985a), may be the determining factor in the ensuing developmental process (Campione-Piccardo *et al*, 1985b).

Retinoic acid need only be present for 6 hours to induce significant differentiation in synchronized EC cultures, but a maximal response requires 24 hour exposure (Rayner and Graham, 1982). This suggests that EC cells must pass through at least one, and possibly more, cell cycles in the presence of the drug before they become developmentally reprogrammed. Ogiso *et al* (1982) have presented evidence that early stages in the differentiation of F9 are reversible on removal of retinoic acid. These observations imply that the inductive interaction of retinoids with EC cells is either time-dependent, or that it occurs only during a specific phase in the cell cycle. Further studies on synchronized cells, or ideally on cultures subjected to a reversible growth arrest, are required to resolve these two possibilities.

Differentiation of EC cells is induced by retinoic acid in defined medium containing insulin, transferrin and fibronectin instead of serum (Rizzino and Crowley, 1980). Therefore retinoid activity is not dependent on interaction with other serum components.

Retinoids have profound biological effects other than their actions on teratocarcinoma cells (Wolbach and Howe, 1925; reviewed by Lotan, 1980; Sporn and Roberts, 1983). Their systemic actions are hormone-like, effecting regulation of the cellular differentiation and proliferation of both epithelial and mesenchymal tissues. They are essential for normal embryonic development. They also suppress the process of carcinogenesis in experimental animals,

which has fostered considerable research into their therapeutic potential. Retinoic acid can substitute for the major physiological retinoid, retinol (vitamin A), in most capacities, but not in the maintenance of the visual system (where retinal, obtained by oxidation of retinol, forms part of the pigment rhodopsin) or of reproductive function (Dowling and Wald, 1960; Thomson *et al*, 1969). This implies that retinoic acid cannot be converted to retinol *in vivo*, and that the modes of action of the two compounds may not be identical at the molecular level.

Several hypothetical mechanisms of retinoid action have been postulated (reviewed by Lotan, 1980; Sporn and Roberts, 1983; Jetten, 1984). Retinoids are amphipathic molecules (Fig.1.3) which can partition directly into cell membranes. This could perturb membrane structure and modify permeability and the activities of membrane-bound enzymes (Jetten, 1984). Retinoids have been shown to reduce the apparent microviscosity of erythrocyte and teratocarcinoma cell membranes (Meeks *et al*, 1981; Jetten *et al*, 1982). However, this effect, along with other indications of altered physicochemical properties of cell membranes, shows little structural specificity and is only manifest at high concentrations (10^{-5}M or greater). Membrane labilization probably underlies the cytotoxic effects of such grossly unphysiological levels of retinoids (Lotan, 1980), and direct effects on the plasma membrane may play a role in their inhibition of intercellular communication (Section 1.8.). Such a mechanism is unlikely to be involved in the systemic actions of retinoic acid *in vivo*, or in the induction of differentiation of teratocarcinoma and other malignant stem cells *in vitro*, however, as these occur at concentrations of 10^{-7}M or less and exhibit strict structural requirements (see below).

A second proposed primary role of retinoids is to modify membrane glycoproteins via participation in sugar transfer reactions (De Luca, 1977). Resultant changes in cell surface properties or secreted products could modulate cell growth and differentiation. Retinoids do alter cell membrane glycoconjugates (De Luca, 1977; Lotan, 1980; Lotan *et al*, 1983) and the direct involvement of retinol in glycosyl transfer, via the intermediate mannosyl retinyl phosphate, has been demonstrated (De Luca, 1977). However, there is no direct evidence for the existence of a corresponding metabolite derived from retinoic acid and the latter cannot be converted to retinol *in vivo* (Dowling and Wald, 1960; Sporn and Roberts, 1983). Changes in cell surface glycosylation patterns of murine melanoma cells exposed to retinoic acid take place over a period of

days following an initial lag period and appear to result from enhanced glycosyltransferase activity, possibly arising from *de novo* transcription (Lotan *et al*, 1984). Alterations in cell surface glycoprotein expression by EC cells treated with retinoic acid, for example loss of SSEA-1, occur in sequence with other changes associated with differentiation and appear to be a consequence rather than a cause of cell determination (Ogiso *et al*, 1982).

Attention to the mechanism of induction of EC cell commitment by retinoic acid and its analogues has therefore focussed mainly on a third hypothesis. This is that retinoids act in a similar manner to steroid hormones by binding to receptors present in target cells to form complexes which directly regulate gene expression via association with chromatin (O'Malley and Means, 1974). Specific intracellular receptors for retinol and retinoic acid have been characterized (Bashor *et al*, 1973; Ong and Chytil, 1975; reviewed by Chytil and Ong, 1978). Cellular retinol binding protein (CRBP) and cellular retinoic acid binding protein (CRABP) differ in both their ligand specificities and tissue distributions. CRABP is detectable on sucrose gradient centrifugation of tissue preparations incubated with [^{3}H]-all-*trans*-retinoic acid as a saturable binding component with a sedimentation coefficient of 2**S**. It does not bind retinol or retinal and is specific for retinoic acid and analogues which retain the terminal carboxyl group (Fig.1.3). Rat CRABP is a single polypeptide with a molecular weight of 14,600. It is present in most foetal tissues but has a more limited distribution in the adult. Changes in tissue levels of CRABP during perinatal development and in the induction of neoplasia suggest that it may be involved in the control of cellular proliferation and differentiation (Chytil and Ong, 1978).

The proposed interaction of retinoic acid-CRABP complexes with nuclear components is based largely on the results of studies on the mode of action of retinol (Takase *et al*, 1979; Liau *et al*, 1981; Porter *et al*, 1983). These have shown that CRBP is localized mainly in the cytoplasm and that binding of retinol to this receptor is essential for its delivery to the interior of the nucleus. There the retinoid associates with specific chromatin binding sites and CRBP returns to the cytoplasm (this is different to the situation with steroid hormones where the intact hormone-receptor complex binds to nuclear acceptor sites). Its functional significance has yet to be established, but the specific interaction between retinol and chromatin may affect gene expression. Nuclear translocation of retinoic acid likewise appears to be dependent on association with binding protein in the cytoplasm (Wiggert *et al*, 1977; Jetten

and Jetten, 1979; Mehta *et al*, 1982). However, there is controversy over the presence of unbound CRABP in nuclei and the persistence of nuclear retinoic acid-CRABP complexes. This may reflect differences in detail between the modes of action of retinol and retinoic acid, but little if any indication of the purity of nuclear preparations is given in many reports and it is possible that apparently conflicting observations might be reconciled by the application of a more rigorous methodology. As with retinol and CRBP, a causal relation between the association of retinoic acid with CRABP and a given cellular response has yet to be established, though there is considerable circumstantial evidence in favour of such a mechanism.

EC cells possess CRABP (Jetten and Jetten, 1979; Matthaei *et al*, 1983) and the effectiveness of various retinoids in inducing differentiation parallels their affinity for the binding protein (Jetten and Jetten, 1979; Jones-Villeneuve *et al*, 1983). Recently a new series of retinoids, retinoidal benzoic acid derivatives or arotinoids, have been developed. The potency of these compounds as inducers of EC cell differentiation appears dependent on their steric and biochemical proximity to all-*trans*-retinoic acid (Strickland *et al*, 1983) and their capacity to bind to CRABP (Sherman *et al*, 1983b).

Retinol, which is not bound by CRABP, can induce differentiation of some EC lines (Sherman *et al*, 1983a). However, this effect is only manifest at 100-1000-fold higher concentrations than retinoic acid, suggesting that activity may be a consequence of oxidation of the alcohol to the acid (Fig.1.3).

Schindler *et al* (1981) isolated two variant EC clones which fail to differentiate in retinoic acid. These cell lines do not possess significant levels of CRABP. Subsequently a third non-differentiating variant which lacks CRABP was obtained in the same laboratory (McCue *et al*, 1983). This is highly suggestive of a role for CRABP in the inductive pathway. However, the caveat should be entered that these three variants are all derived from the same parental line, PCC4aza1R, which was originally selected by continuous culture in retinoic acid (Jetten *et al*, 1979) and may therefore show an abnormal responsiveness at both the cellular and the molecular level. With the exception of one of a series of F9 derivatives, which shows greatly reduced expression of CRABP (Wang and Gudas, 1984), other non-differentiating variants isolated from different parental lines exhibit normal levels of CRABP (McCue *et al*, 1983; Wang and Gudas, 1984; Moore *et al*, 1985). This does not constitute evidence against the

proposed function of CRABP, however, as it is possible that these variants are defective either in some post-receptor phase of the pathway of induction or in the process of cytodifferentiation. The latter appears to be the case for the F9-5C clone isolated by Moore *et al* (1985) which undergoes some differentiative changes on exposure to retinoic acid but does not progress to the fully differentiated phenotype.

Complementation analyses of a series of differentiation-defective EC clones (McCue *et al*, 1984a) indicate that non-responsiveness to chemical inducers is a recessive character and are consistent with mediation of retinoic acid action by CRABP. Thus hybrids formed between two variants which lack CRABP showed restored sensitivity to retinoic acid and expressed low, but detectable, levels of CRABP (implying that the structural gene is retained by at least one of the variants). Hybrids formed by fusions of cells from the same variant clone retained their differentiation-defective phenotype and possessed little or no CRABP. Furthermore, McCue *et al* (1984b) have shown that exposure of one of the CRABP-deficient lines to sodium butyrate (see below) leads to the reappearance of high levels of CRABP and concomitantly of responsiveness to retinoic acid.

There is thus a substantial body of evidence consistent with an essential role for CRABP in the retinoid-induced differentiation of EC cells. Responsiveness to retinoic acid also corresponds with the presence or absence of retinoic acid in several other systems (Lotan, 1980; Haussler *et al*, 1983; Ueda *et al*, 1985). However, there are some cells which apparently lack CRABP yet retain sensitivity to retinoic acid (Sporn and Roberts, 1983; Jetten, 1984). Notable amongst these is the HL60 human promyelocytic leukaemia cell line. HL60 undergoes terminal granulocytic differentiation when treated with retinoic acid. CRABP is not detectable by sucrose gradient analysis of cytosol fractions prepared from these cells (Douer and Koeffler, 1982). This procedure is not very sensitive, however, so the conclusion that HL60 and certain other cell lines completely lack CRABP remains somewhat tentative until a more precise assay is developed. Nonetheless these findings have led to further hypotheses on the mode of action of retinoids. In particular there is interest in the interactions between retinoic acid and protein kinases (Sporn and Roberts, 1983) which have been implicated in the control of cell growth and differentiation by a variety of studies (reviewed by Friedman, 1976; Nishizuka, 1984).

Increased activity of cyclic AMP-dependent protein kinase appears to be one of the first detectable responses of F9 EC cells to retinoic acid, occurring within 3-15 hours (Plet *et al*, 1982). This precedes morphological and other biochemical indications of differentiation and, given the pronounced effects of elevated levels of cyclic AMP on this system (see previous Section), may be involved in mediating some of the effects of retinoid treatment. However, agents such as dibutyryl cyclic AMP, cholera toxin and phosphodiesterase inhibitors, which increase intracellular cyclic AMP and promote retinoic acid-induced differentiation of F9, do not induce any differentiative response in the absence of retinoic acid. Therefore enhanced activity of cyclic AMP-dependent protein kinase is not a sufficient condition for cell commitment. Moreover, it is not clear whether retinoic acid interacts directly with the kinase or whether it enhances activity via *de novo* transcription, possibly mediated via CRABP. Finally, Wang and Gudas (1984) report that they can detect no change in cyclic AMP-dependent protein kinase activity on treatment of their F9 cells with retinoic acid.

Calcium-and phospholipid-dependent, cyclic AMP-independent protein kinase (protein kinase C) activity in F9 only becomes significant 3-5 days after exposure to retinoid, coinciding with the onset of overt cytodifferentiation (Kraft and Anderson, 1983; Anderson *et al*, 1983). P19 EC cells in contrast possess high levels of this activity (Snoek *et al*, in press). The finding that phorbol 12-myristate 13-acetate (PMA), which activates protein kinase C, potentiates the retinoic acid-induced differentiation of these cells (Snoek *et al*, in press), suggests that this enzyme may have an important function in the differentiation process. PMA alone, however, whilst it causes transient morphological changes, does not induce differentiation of P19. Furthermore, a direct effect of retinoic acid on protein kinase C has not yet been demonstrated.

The relationship between retinoic acid, protein kinase activity and cellular differentiation thus requires further clarification. Available evidence indicates that protein kinases may play an important role in modulating the transition from the committed state to the overtly differentiated phenotype. Their interaction with growth factors and intracellular calcium (Nishizuka, 1984) could conceivably determine the direction of differentiation of EC cells whose commitment has been induced by retinoic acid.

Retinoids reduce activity of the enzyme ornithine decarboxylase (ODC) in a

variety of cell types and suppress its induction by growth factors and tumour promoters (Russell and Frasier-Scott, 1983; Jetten, 1984; Jetten and Shirley, 1985). ODC catalyses the first and rate-limiting step in polyamine biosynthesis. The precise functions of polyamines are obscure but they have been implicated in the regulation of cellular proliferation and differentiation. ODC activity and polyamine levels fall on retinoid-induced differentiation of EC cells (Jetten and Shirley, 1985; Schindler *et al*, 1985; Kelly *et al*, 1985).

Evidence has been presented that retinoids may inhibit ODC via activation of transglutaminase (Russell and Frasier-Scott, 1983). The latter can inactivate ODC by conjugating it with putrescine. The discovery that 2-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, can induce EC cell differentiation (Schindler *et al*, 1983) appears to strengthen the argument for mediation of retinoid action via inhibition of ODC. However, the effect of DFMO on differentiation is abolished by exogenous application of the polyamine putrescine, whereas the action of retinoic acid is unaffected despite an increase in intracellular polyamines (Jetten and Shirley, 1985). Moreover, Nulli-1 cells, which differentiate on exposure to retinoic acid, exhibit reduced polyamine levels on DFMO treatment but remain undifferentiated (Kelly *et al*, 1985). No significant change in transglutaminase activity is observed in retinoic acid-treated F9 cells, nor is any inhibitory activity detectable in cell lysates. The evidence is consistent with a suppression of synthesis of ODC by retinoic acid (Jetten an Shirley, 1985). This does not seem to be an essential step in the inductive mechanism, though it is apparently a sufficient condition for the induction of differentiation of some EC lines.

The latter observation indicates that there is more than one pathway for inducing EC cell differentiation. This conclusion is confirmed by the finding that EC cells can differentiate in defined medium lacking retinoids (Darmon *et al*, 1982; Rizzino, 1983a). Furthermore, some of the variants isolated by McCue *et al* (1983) for non-responsiveness to chemical inducers retain the ability to differentiate in tumours. This implies that mechanisms of cell commitment and differentiation different from those induced by chemical treatment *in vitro* may operate *in vivo*.

Very little is known concerning the mode of action of other chemical promoters of EC differentiation, which are all non-physiological (see previous Section). Butyrate causes hyperacetylation of histones, which may lead to alterations in

chromatin structure and hence in gene expression (McCue *et al*, 1984b). Such effects might be expected to be relatively non-specific, which may explain why only a few EC lines (those intrinsically predisposed to differentiate?) exhibit a differentiation response to butyrate. Retinoic acid (and HMBA) does not induce alterations in histone profiles (McCue *et al*, 1984b; Levine *et al*, 1984). Indeed butyrate has been reported to inhibit the retinoic acid-induced differentiation of F9 (Levine *et al*, 1984). Poly(ADP)ribose synthetase activity may also affect chromatin structure, which could account for the effect of inhibitors of this enzyme in promoting differentiation of the unstable EC-A1 EC cell line (Ohashi *et al*, 1984).

The mechanism of action of HMBA and other Friend cell inducers is completely obscure, both in Friend cells and EC cells. McCue *et al* (1983) have found that four differentiation-defective variants selected for non-responsiveness to retinoic acid also fail to differentiate when treated with HMBA. Furthermore, one of several clones selected for resistance to HMBA is also unresponsive to retinoic acid. This implies that HMBA interacts at some stage with the retinoid pathway. HMBA does not bind to CRABP, nor facilitate its interaction with retinoic acid (McCue *et al*, 1983), but it may enhance its activity in some other way (Sherman *et al*, 1983a).

1.6. PLURIPOTENT CELL LINES ESTABLISHED DIRECTLY FROM EMBRYOS (EK CELLS)

Embryonal carcinoma cells have proved useful tools in the experimental investigation of murine embryogenesis but they do have limitations, as discussed in Section 1.3.4. It is now possible to isolate pluripotential cells into tissue culture directly from normal mouse embryos (reviewed by Evans and Kaufman, 1983). These EK cells should provide a more advantageous system for both molecular and genetic analyses of developmental processes. As some EK lines are capable of forming functional germ line chimaeras (Bradley *et al*, 1984), they also afford the prospect of generating genetically altered mice. This is an objective which has proved elusive using EC cells because only one *in vitro* line, Mett-1, has been demonstrated to colonise the germ-line (Stewart and Mintz, 1981; see Section 1.3.5.). The advantage of employing EK cells compared with direct transformation of the blastocyst (Constantini and Lacy, 1981) is that the former are accessible to complex genetic manipulation and

characterisation before their incorporation *in vivo*.

Initial reports on the isolation of EK cells utilised giant blastocysts generated by implantational delay to increase the number of epiblast cells (Evans and Kaufman, 1981) (though it has since been found that the number of ICM cells is not increased in such blastocysts, A.Handyside, personal communication), or medium conditioned by EC cells to facilitate growth of embryonic stem cells (Martin, 1981). Subsequently, it has been demonstrated that neither of these procedures is necessary (Robertson *et al*, 1983; Axelrod, 1984). Euploid pluripotent stem cell lines can be derived from individual pre-implantation embryos by culture of intact blastocysts or immunosurgically isolated ICMs in microlitre volumes of serum-containing medium (Axelrod, 1984). However, in this, as in earlier procedures, the embryo cells must be maintained on feeder layers of growth-arrested embryonic fibroblasts. EK cells are thus analagous to feeder-dependent EC cells (Section 1.3.3.).

Continuous embryonic stem cell lines have now been established from a variety of inbred and outbred strains of mice. Homozygous diploid lines of parthenogenetic origin have also been isolated (Kaufman *et al*, 1983). EK cells can be obtained bearing homozygous lethal developmental mutations. Thus Magnuson *et al* (1982, 1983) have isolated EK lines homozygous for the t^{w5} mutation of the *t* complex. Interestingly these cells differentiate extensively *in vitro* and in tumours.

In many respects - morphology, cell surface antigen expression, patterns of protein synthesis, differentiative behaviour *in vitro* and *in vivo* - EK cells are indistinguishable from EC cells (Evans and Kaufman, 1983). In particular they give rise to teratocarcinomas when injected subcutaneously into syngeneic mice. This appears to confirm the analogy between EC cells and epiblast cells of the early post-implantation embryo (Section 1.3.4.) and indicates that the former are an appropriate system for the study of specific aspects of cellular differentiation and cell biology. However, the advantages of EK cells, in terms of their isolation from normal embryos, variety of genotypes, retention of euploidy and greater contribution to chimaeras, suggest that in many respects they will supersede tumour-derived cells as a model for early events in mouse embryogenesis, notably in the field of developmental genetics. The principal disadvantage of EK cells, shared by many pluripotential EC cells, is their dependence on feeders which introduces logistical and experimental constraints

into their manipulation in culture (see Chapter 8 for further discussion).

1.7. FACTORS REGULATING THE PROLIFERATION AND DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS

It is apparent that the cues in extracellular environment modulate the differentiation of EC cells (see Sections 1.4. and 1.5.). The study of such developmental signals and their mode of action is hindered by the use of medium containing serum, which is highly complex and ill-defined (reviewed by Rizzino, 1983b; Heath, 1983). Serum contains factors necessary for EC cell viability and proliferation but may also contain activities which stimulate or inhibit differentiation, block particular developmental pathways and/or promote others, interact with chemical inducers, obscure the actions of endogenous factors produced by EC cells and their differentiated progeny, and confer selective growth advantages on specific cell types. The development of defined serum-free medium for EC cells (Rizzino and Sato, 1978; Rizzino and Crowley, 1980; Heath and Deller, 1983) should facilitate the identification, both of factors essential for survival and proliferation, and of activities regulating differentiation. It may also help clarify the mode of action of inducing agents.

Growth of the quasi-nullipotent feeder-independent EC lines F9 and PC13 can be sustained by basal medium supplemented with a cell attachment factor (fibronectin or laminin), a hormone (insulin) and an accessory factor (transferrin) (Rizzino and Crowley, 1980; Rizzino, 1983b; Heath and Deller, 1983). This medium is known as EM-3. Differentiation of both cell lines in EM-3 can be induced by the addition of retinoic acid. The differentiated derivatives obtained appear identical to those obtained in serum-containing medium. Growth is density-dependent in EM-3, however, and there are conflicting reports as to whether it is possible to achieve clonal multiplication via the addition of lipoproteins (Rizzino, 1983b; Heath and Deller, 1983).

The functions of fibronectin and transferrin in EM-3 are well-defined (Heath, 1983). Fibronectin need not be present in the medium if dishes are precoated with this macromolecule (though a role additional to that in cell attachment is indicated by its induction of differentiation of multipotent EC lines, Rizzino, 1983a,b) and transferrin has been shown to mediate the uptake of extracellular iron by EC cells via a high affinity transferrin receptor system. The role of

insulin is less clear as PC13 EC cells lack insulin receptors (Heath *et al*, 1981). Nagarajan *et al* (1982) showed that the very high concentrations of insulin employed in EM-3 (1µg/ml) could be substituted by physiological levels (50-100ng/ml) of rat insulin-like growth factor-II (IGF-II, also known as multiplication-stimulating activity or MSA). They further demonstrated that EC cells possess specific cell surface receptors for IGF-II. However, insulin does not cross-react with the IGF-II receptor (Massague and Czech, 1982) and does not compete with IGF-II for binding to PC13 EC cells (Heath, 1983). Its mode of interaction with PC13 EC cells therefore remains unresolved.

Subsequently, Heath and Deller (1983) found that neither insulin nor IGF-II are required by PC13 if EM-3 is supplemented with high and low density lipoproteins. This medium is termed ECM. Lipoproteins appear to meet a lipid requirement for EC cell multiplication. The possession of high affinity receptors for low density lipoproteins by EC cells sugests that they may represent the physiological system for supplying lipids (Heath, 1983). Insulin and IGF-II can presumably compensate for this demand via their actions on lipid biosynthesis.

Heath (1983) has pointed out that essential components of ECM are synthesized *in vivo* by visceral endoderm. Both transferrin (Adamson, 1982) and fibronectin (Hogan, 1980) are produced by the visceral endoderm of the post-implantation embryo. This tissue may also be a source of lipoproteins (Heath, 1983) and IGF-II (Anderson *et al*, 1983). The visceral embryonic endoderm immediately overlies the epiblast at the egg cylinder stage of development (Section 1.2.2.) and the above observations raise the possibility that maintenance of embryonic stem cells in utero may be dependent on factors produced by their differentiated progeny. This idea is consistent with the earlier finding that primitive ectoderm cells do not proliferate in isolation from visceral endoderm, unless they are provided with a feeder layer (Hogan and Tilly, 1977).

A second interesting feature of ECM is that it contains no growth factors. Proliferation of PC13 is apparently independent of exogenous growth factors. This probably accounts for the finding that the multiplication rate of PC13 is impervious to modulation by serum factors (Heath, 1983). It implies that the sole means of regulating EC cell proliferation may be via differentiation. Differentiated derivatives do exhibit growth factor requirements and some have finite proliferative lifespans (Rees *et al*, 1979; Heath, 1983; Rayner and Pulsford,

1985). However, EC cells and particularly those which are feeder-independent, have been subjected to considerable selective pressure for autonomous growth (see Section 1.3.). It is possible that the growth factor-independent phenotype of PC13 is a consequence of such selection rather than a property of malignant teratocarcinoma cells, much less embryonic stem cells.

The proposition that pluipotent embryonic cells may proliferate autonomously in the presence of factors essential for cell viability, and that their rate of population expansion is therefore regulated by differentiation is difficult to test experimentally. It does predict that EK cells should grow in serum-free conditions defined for EC cells. The difficulty here is that EK cells are feeder-dependent. The interactions between feeders and EC (or EK) cells are complex (Isacke and Deller, 1983) but it appears that their effect on differentiation is at least partly mediated via a diffusible factor or factors secreted into the medium (Smith and Hooper, 1983; Koopman and Cotton, 1984). Thus at least one additional activity to those present in ECM is required for maintenance of pluripotent feeder-dependent stem cells. This could be a growth factor which inhibits differentiation indirectly by promoting continuous stem cell proliferation, if interrupted or slowed multiplication can trigger cell commitment. Alternatively it might directly inhibit the differentiation process. Factors which inhibit differentiation may also be present in serum as Rizzino (1983c) reports that the proportion of OC15S1 EC cells induced to differentiate by plating on fibronectin-or laminin-coated dishes is greater in defined than in serum-containing medium.

The production of another vital activity by feeder cells is evidenced by the observation that PC13 does not grow at clonal density in ECM except in the presence of feeder cells (Heath and Deller, 1983). This effect is not reproduced by conditioned medium, so appears to be distinct from the inhibitory action of the latter on differentiation of feeder-dependent EC cells.

It would thus be somewhat premature to conclude that pluripotent EC cells, let alone embryonic stem cells, are not subject to exogenous mechanisms of growth control. However, the development of defined medium for these cells has allowed the identification of some endogenously produced growth factors (reviewed by Heath, 1983). If some of these factors function in an autocrine manner, as appears to be the case for some other malignant cells (Waterfield *et al*, 1983; Sporn and Roberts, 1985), it could account for the apparent

independence of EC cells from exogenous regulation. In this regard it is noteworthy that PC13 and F9 EC cells produce a transforming growth factor (TGF) activity which induces colony formation in semi-solid medium by normal rat fibroblasts (Rizzino *et al*, 1983; Rizzino, 1983c; Heath, 1983). This activity appears to be distinct from the two characterized transforming growth factors (De Larco and Todaro, 1978); TGF-α which is an EGF-like activity produced by a variety of neoplastic cells, and TGF-β which is released by normal tissues and virally transformed cells (for review see Sporn and Roberts, 1985). Both these factors have been shown to function in an autocrine mode. The TGF produced by EC cells has not been purified but it does not compete with EGF for binding to membrane receptors, in contrast to TGF-α, and unlike TGF-β its ability to promote anchorage-independent growth of NRK rat fibroblasts is not dependent on EGF (Rizzino, 1983c). Whether it might have an autocrine function cannot be determined until purification and the performance of radioreceptor assays and/or immunological inhibition studies, as there is no appropriate bioassay.

An indication that EC cells may produce a factor or factors which act upon themselves is provided by reports that medium conditioned by PSA1 EC cells facilitates the establishment of EK lines (Martin, 1981; Martin and Lock, 1983). However, the protocol employed by these workers entails culturing the feeder-dependent PSA1 cells for 4 days in the absence of feeders, during which time differentiative changes may occur, including alterations in the production of growth factors (Heath, 1983).

Gudas *et al* (1983) have reported that PSA1 cells produce an activity related to platelet-derived growth factor (PDGF) but do not themselves bind exogenous PDGF. The latter property could arise from occupation of receptors by endogenous ligand (possibly also resulting in receptor down-regulation), or from a lack of receptors. Recently Rizzino and Bowen-Pope (1985) have repeated these observations on F9 and PC13 and further demonstrated that differentiation is accompanied by the appearance of functional PDGF receptors and by a decrease in the secretion of PDGF-like activity. This indicates that EC cells may produce factors which promote proliferation of their differentiated progeny, as previously proposed by Heath and co-workers (Heath and Isacke, 1983; Isacke and Deller, 1983; Heath, 1983; Heath and Isacke, 1984). The precise nature of this PDGF-like factor is unclear, however, as although it competes with I^{125}-PDGF for binding to membrane receptors, it is not antigenically

identical to PDGF (Rizzino and Bowen-Pope, 1985). Moreover, Heath and Isacke (1984) report that PC13 does not express PDGF-related mRNAs.

The only growth factor produced by EC cells which has so far been purified to homogeneity is embryonal carcinoma-derived growth factor (ECDGF) (Heath and Isacke, 1984). ECDGF is a single chain polypeptide of 17,500 daltons produced by PC13 EC cells. It is distinct from other characterised growth factors, both in structure and bioactivity. ECDGF stimulates DNA synthesis by a variety of differentiated cells, including PC13 END (Section 1.5.1.) and specific primary embryonic cell types. It is not known whether it acts in an autocrine manner with undifferentiated PC13 cells.

The observation that ECDGF supports proliferation of embryonic and embryo-derived differentiated cells (Heath and Isacke, 1984), coupled with its production by a cell line believed to be analogous to early embryonic stem cells (Section 1.3.3.), suggests that it may have an important function in embryonic development. Other growth factors for which there is evidence of a role in early embryogenesis include transforming growth factors (Proper *et al*, 1982; Lawrence *et al*, 1984; Hill *et al*, in press) and IGF-II (Moses *et al*, 1980a; Anderson *et al*, 1983b; Adams *et al*, 1983). Embryonic growth factors may be involved not only in the regulation of cellular proliferation, but also, via their interaction with protein kinases (reviewed by Nishizuka, 1984) in the control of differentiation (see discussion in Section 1.5.2. on the possible role of protein kinases in the mediation of retinoic acid-induced differentiation of EC cells).

A direct demonstration that growth factors can modulate EC cell differentiation has been provided by Liesi *et al* (1983). These workers showed that culture of low density monolayer cultures of F9 for 10 days or more in the presence of retinoic acid, dibutyryl cyclic AMP and nerve growth factor resulted in adrenergic neuronal differentiation, as detected by the expression of neurofilaments and the appearance of immunoreactivity for tyrosine hydroxylase and Leu-enkephalin-like peptides. The presence of nerve growth factor was essential for the latter two developments, though it had no effect in the absence of either or both retinoic acid and dibutyryl cyclic AMP.

Many oncogenes encode products related to growth factors or their receptors, or involved in the post-receptor pathway of signal transduction (Waterfield *et al*, 1983; reviewed by Sporn and Roberts, 1985). The cellular homologues of

these genes are believed to be active during embryonic development where they may regulate cellular proliferation and differentiation. Consequently there is considerable interest in the expression of oncogene-related gene transcripts by EC cells and early embryos (Muller, 1983; Campisi *et al*, 1984; Sejerson *et al*, 1985; Jakobovits *et al*, 1985). This may even prove to be a means of identifying embryonic growth factors and clarifying their role.

Recently Muller and Wagner (1984) have found that DNA-mediated gene transfer of the c-*fos* proto-oncogene into F9 EC cells results in differentiation of those tranfectants which express the c-*fos* messenger RNA and protein. Moreover, the differentiated cell type obtained is distinct from the parietal endoderm-like cells formed following exposure to retinoic acid (Section 1.5.1.). The developmental significance of this finding is unclear but it demonstrates that proto-oncogenes can modulate cellular differentiation.

1.8. METABOLIC COOPERATION IN EMBRYONAL CARCINOMA CELLS

Metabolic cooperation is the exchange of molecules between cells through permeable junctions formed at sites of cell contact (reviewed by Hooper and Subak-Sharpe, 1981; Hooper, 1982a). This intercellular transfer is mediated via specialized membrane structures known as gap junctions (Finbow, 1982). Gap junctions function as molecular sieves which exclude molecules of molecular weight greater than 1,000 in mammals (somewhat larger in invertebrates). Junctional communication is thus limited to ions, low molecular weight metabolites and other small molecules. Gap junctions are found in most animal tissues. In excitable tissues they provide pathways for the intercellular transmission of electrical impulses but their functions elsewhere are ill-defined (Hooper and Subak-Sharpe, 1981). They probably have an important role in tissue homeostasis, but could also be involved in modulation of growth and development.

Gap junctions are widely distributed in developing systems and junctional communication could provide a mechanism by which cell interactions regulate developmental processes (Furshpan and Potter, 1968; Hooper, 1982b). Gap junctional channels could allow the transfer of signal molecules influencing differentiation and/or morphogenesis. Conversely, the absence of functional junctions would present a barrier to such transfer. Recently evidence has been

presented that genetically defined developmental compartments in insects may correspond to gap junctional communication compartments, suggesting that junctional transfer may be involved in compartment formation and the control of morphogenesis (Lo, 1982; Blennerhassett and Caveney, 1984; Weir and Lo, 1984).

Junction-mediated intercellular communication is first unequivocally detected in the mouse embryo at compaction (Section 1.2.1.) (Lo and Gilula, 1979) and gap junctions are subsequently found in most embryonic tissues, making them suitable candidates for the transmission of developmental signals. Experimental investigation of the possible functions of metabolic cooperation in embryogenesis is facilitated by the use of embryonal carcinoma cells (reviewed by Hooper, 1982b). In particular it is possible to isolate variants with altered junctional communication properties whose developmental capacity *in vitro* and *in vivo* can then be characterised.

Somatic cell genetic analysis of metabolic cooperation is made possible by the existence of selective systems for the isolation of communication-defective variants and communication-competent revertants (Hooper and Subak-Sharpe, 1981) and by the availability of assays for monitoring the efficiency of junctional communication *in vitro* (Pitts and Simms, 1977; Hooper, 1982b). Cooperation-defective cells can be selected for by the thioguanine "kiss of death" system. In this procedure HGPRT$^-$ cells are cocultured with an excess of wild- type cells at high cell density in the presence of the toxic purine analogue 6-thioguanine. Wild-type cells are killed due to their metabolism of 6-thioguanine into the nucleotide form and hence its incorporation into nucleic acids. HGPRT$^-$ cells cannot metabolize 6-thioguanine but those communicating with wild-type cells are killed due to transfer of the toxic nucleotide. The metabolic cooperation-defective (mec$^-$) PC13TG8 derivative R5/3 was isolated in this manner (Slack *et al*, 1978).

This procedure was also employed by Smith (1984) in the selection of the mec$^-$ variant PT2md1 used in this study. PT2md1 is derived from a subclone, PT2, of PSA4TG12, a HGPRT$^-$ variant of PSA4 (Slack *et al*, 1977). PT2 is feeder-dependent and the selection was therefore performed in the presence of STO feeders, which are also HGPRT$^-$. PT2md1 displays the poorest communication ability of a series of such variants (Smith, 1984) as measured by two separate assays. The first is the capacity to transfer radiolabelled

nucleotides which can be detected by autoradiography (Pitts and Simms, 1977). The second is an indirect assay of the ability to participate in exchange of alkali metal ions, based on rescue from ouabain toxicity by ouabain-resistant STO feeders (Hooper and Morgan, 1979). In addition to its mec$^-$ phenotype, PT2md1 also shows greatly reduced developmental capacity in embryoid bodies (Smith, 1984). It is unclear, however, whether there is any causal connection betweeen these phenomena.

High concentrations of retinoic acid have been shown to inhibit intercellular communication (Pitts *et al*, 1981; Hamilton and Pitts, 1981). This effect is manifest in all cell types tested, including embryonal carcinoma (Pitts *et al*, in press; Smith, 1984). It is rapid and reversible and apparently mediated via the closure of gap junctions (Pitts *et al*, in press). The relationship between inhibition of metabolic cooperation and other actions of retinoic acid is uncertain. As discussed above it is conceivable that blocking junctional communication could interfere with normal developmental signals. This has been postulated to underlie the profound perturbations of pattern formation in developing and regenerating limbs caused by retinoic acid (Maden, 1982).

PTmr0, the other metabolic cooperation variant investigated in this study, is resistant to the effects of retinoic acid on junctional communication (Smith, 1984). It was selected from PSA4TG12 by a modification of the ouabain "kiss of life" procedure (Hooper, 1982b). The latter is simply an extension of the ouabain rescue assay for detecting metabolic cooperation (see above). Communication-competent cells are rescued from the toxic effects of ouabain via exchange of alkali metal ions with ouabain-resistant cells. PTmr0 was isolated by culture of PSA4TG12 on ouabain-resistant STO feeders in the presence of ouabain and retinoic acid (Smith, 1984). Uridine nucleotide transfer assays revealed that it is resistant to inhibition of junctional communication by retinoic acid.

1.9. OUTLINE OF THIS INVESTIGATION

The work reported in this thesis can broadly be divided into two related areas, corresponding to the first and second halves respectively of the experimental results section (Chapters 3 to 7). Both are essentially concerned with the processes of commitment and differentiation in pluripotent EC cells and by

analogy in embryonic stem cell populations. Chapters 3 and 4 examine some biological and biochemical aspects of the interactions between EC cells and the differentiation-inducing chemical retinoic acid. This is primarily a study of the interrelationship between the induction of differentiation and two other effects of retinoic acid, namely inhibition of metabolic cooperation and increased cell mortality. It It is also an effort to determine whether there is a single unifying biological mode of action of retinoic acid, or whether multiple mechanisms exist. As such it represents a minor contribution to the investigation of retinoids, which currently ranges from the molecular to the clinical level. In Chapters 5 and 6 by contrast, a novel activity which inhibits rather than induces EC cell differentiation is described. The biological effects of this activity are characterised in Chapter 5 and its partial purification and relation to known growth factors are reported in Chapter 6. The experimental significance and theoretical implications of this discovery are discussed. Finally, in Chapter 7 the findings of a preliminary investigation into the dependence of some EC lines on the thiol compound 2-mercaptoethanol and its possible involvement in the inhibition of differentiation are reported.

CHAPTER 2

MATERIALS AND METHODS

2.1. CELL LINES

The STO mouse fibroblast (Martin and Evans, 1975b) is a thioguanine-resistant and ouabain resistant derivative of a continuous line derived from embryos of strain SIM mice (Ware and Axelrad, 1972). Swiss 3T3 mouse fibroblasts (Todaro and Green,1963) were provided by J. Boyd, Dept. of Surgery, Edinburgh University Medical School. Don is a Chinese hamster cell line isolated from the lung of a normal adult male Chinese hamster (Hsu and Zenzes, 1964).

Buffalo rat liver (BRL) cells are derived from a clone isolated from the liver of a five week old Buffalo rat (Coon, 1968) and were obtained from J. Pitts, Beatson Institute, Glasgow. The subclone BRL-3A2 (Nissley *et al*, 1977) was supplied by S.P. Nissley, National Cancer Institute, Maryland, USA.

BSC-1 is an epithelial cell line of African green monkey kidney origin (Hopps *et al*, 1963). NRK 49F is a clone of normal rat kidney fibroblasts (De Larco and Todaro,1978a) obtained from J. Heath, Dept. of Zoology, Oxford University.

The origins and phenotypes of the EK and EC lines used in this study are tabulated in Table 2.1.

2.2. CHEMICALS AND MATERIALS

Chemicals were purchased from Sigma Chemical Co. (Poole, Dorset) or BDH (Poole, Dorset), unless otherwise specified. Coomassie Brilliant Blue G-250 dye reagent, charcoal-dextran tablets and Bio-Gel P-10 were obtained from Bio-Rad (Watford, Herts); mitomycin-C and unlabelled retinoids were provided by Calbiochem (Bishops Stortford, Herts), hexamethylenebisacetamide (HMBA) by Aldrich (Gillingham, Dorset), α_2-macroglobulin by Boehringer (Lewes, East Sussex), Liquiscint scintillant by National Diagnostics Ltd. (Aston Clinton, Bucks); and [^{3}H]-thymidine (S.A.=53 Ci/mmol) by Amersham (Amersham, Bucks). All-*trans*-[^{3}H]-retinoic acid (S.A.=30Ci/mmol) was a generous gift from Hoffmann-La Roche (Basel, Switzerland).

Table 2.1
Embryonic Stem (EK) Cells and Teratocarcinoma-Derived Cell Lines Used in this Study

A. Embryonic stem (EK) cell lines

Cell Line	Source	Karyotype	Phenotype*	Reference
B2B2	129/Sv//Ev fertilized blastocyst	Euploid,XY	FD	Robertson *et al* (1983)

B. Embryonal carcinoma (EC) cell lines isolated from tumours

Cell Line	Tumour of Origin	Source of Tumour	Phenotype	Reference
PC13	OTT-6050	6 day embryo	FI	Bernstine *et al* (1973)
SCC-PSA4	OTT-5568	3 day embryo	FD	Martin and Evans (1975a)
SCC-S2	"	"	FD	"
Nulli-SCC1	LS-402C-1684	3 day embryo	FI	"

C. Embryonal carcinoma (EC) cell lines selected from other cell lines

Cell Line	Cell Line of Origin	Selection Procedure	Phenotype	Reference
PC13.5	PC13	cloning	FI	Hooper and Slack (1977)
R5/3	PC13TG8	thioguanine "kiss of death"	FI_R TG^-	Slack *et al* (1978)
PSA4TG12	SCC-PSA4	thioguanine resistance	FD_R TG^-	Slack *et al* (1977)
PTmr0	PSA4TG12	cooperation in retinoic acid	FD_R TG^-_R mec^-	Smith (1984)
PT2	PSA4TG12	cloning	FD_R TG^-	Smith (1984)
PT2md1	PT2	thioguanine "kiss of death"	$FD^{\wedge}_S$ TG^- mec^-	Smith (1984)

D. Differentiated teratocarcinoma-derived cell lines

Cell Line	Tumour of Origin	Source of Tumour	Phenotype	Reference
PYS	OTT-6050	6 day embryo	Parietal endoderm	Lehman *et al* (1975)
PSA5E	OTT-5568	3 day embryo	Visceral endoderm	Adamson *et al* (1977)

*FD, feeder-dependent; FI, feeder-independent; TG^R, resistant to thioguanine toxicity; mec^-, deficient in metabolic cooperation; mec^-_R, resistant to inhibition of metabolic cooperation by retinoic acid

^But see Section 4.5.

Phosphate-buffered saline tablets, Glasgow-modified Eagle's medium concentrate, medium supplements, trypsin and chicken serum were obtained from Flow (Irvine, Ayrshire), and Cytodex-3 microcarriers from Pharmacia (Milton Keynes, Bucks). Calf serum came from Flow, Gibco (Uxbridge, Middx), Sera-labs (Crawley, Sussex), or Northumbria Biologicals (Cramlington, Northumberland).

Multiplication-stimulating activity (MSA) was obtained from Collaborative Research through Uniscience Ltd. (London). Insulin-like growth factor-II (IGF-II), purified to homogeneity from medium conditioned by Buffalo rat liver cells (Marquardt *et al*, 1981) was generously provided by J. Heath, Dept. of Zoology, Oxford University, as was murine epidermal growth factor (EGF). Porcine platelet-derived growth factor (PDGF) was supplied by Bioprocessing Ltd. (Consett, Durham). Transforming growth factor-β (TGF-β), purified to homogeneity from human platelets (Assoian *et al*, 1983), was a gift from A. Strain and D. Hill, Dept. of Paediatrics, University of Sheffield.

Human plasminogen was a gift from Dr. I. MacGregor of the Scottish National Blood Transfusion Service (SNBTS). Bovine fibrinogen (plasminogen and thrombin-free) was supplied by Calbiochem.

Monoclonal antibodies, anti-SSEA-1 (Solter and Knowles, 1978), M1/22-25 (Stern *et al*, 1981) and 2H9 (Stern *et al*, 1983) were a gift from P. Stern, Dept. of Immunology, University of Liverpool.

Radioactivity was measured using a Denley Delta 300 liquid scintillation counter with a counting efficiency for [^{3}H] of 35%. Photography was performed using a Leitz camera.

2.3. CELL CULTURE

Cell culture was carried out in Lux (obtained through Flow), Falcon (from Becton Dickinson, Twickenham, Middx.) or Nunc (via Gibco) disposable plastic flasks and dishes, or on 2.5g/l of Cytodex-3 microcarriers in a Techne microcarrier culture apparatus. Media and cells were transferred using Volac serological pipettes (MacKay and Lynn, Edinburgh).

Pipettes were soaked overnight in Calgonite (100g/l), washed in water and then

0.01M HCl, rinsed thoroughly in tap water followed by distilled water, dried in a hot air oven and sterilized in canisters by heating to 160°C for two hours before use. Glassware for use with microcarriers was first siliconized by rinsing with dimethyldichlorosilane, baking in a hot air oven and washing thoroughly.

Cells were grown in complete medium (CM), which consists of Glasgow-modified Eagle's medium (Stoker and McPherson, 1961) supplemented with non-essential amino-acids (0.1mM each), 1mM sodium pyruvate, 0.1mM 2-mercaptoethanol (Oshima, 1978) and 10% (v/v) bovine serum (selected batches). With the exception of PT2 and PT2md1, EK and EC cells growing on gelatin were cultured in foetal bovine serum, whereas EC cells on feeder layers and other cell types were routinely cultured in newborn calf serum. Cultures were maintained at 37°C in a humidified atmosphere of 95% air, 5% carbon dioxide and were checked periodically for mycoplasma contamination using the method of Chen (1977), modified by the use of human fibroblasts. Any cultures suspected of being contaminated were discarded.

Stocks of PC13 and of its derivatives were grown on plastic surfaces pre-coated with gelatin (Bernstine *et al*, 1973). The plastic was covered with a 0.1% gelatin solution, and incubated at 4°C to allow adsorption on to the surface of the plastic. Excess gelatin was then aspirated away. Stocks of SCC-PSA4 and its derivatives and of the EK line B2B2 were maintained on STO feeder layers prepared as described below (Section 2.3.3.).

2.3.1. Passaging of cells

Cells to be passaged were first washed twice with phosphate-buffered saline (PBSA). This is an aqueous solution containing 8g/l NaCl, 0.2g/l KCl, 1.15g/l Na_2HPO_4 and 0.2g/l KH_2PO_4 . They were then incubated with TVP; a mixture of 0.25g/l trypsin, 0.37g/l disodium EDTA and 10ml/l chicken serum in PBSA. The trypsin was neutralised with medium containing serum. Single cell suspensions were generated when required by passing cells twice through a 23G1 syringe needle (Becton Dickinson). This procedure decreased viability by less than 10% as estimated by dye exclusion and effects on cloning efficiency. Split ratios were generally of the order of 1:10 and no culture was maintained for more than ten serial passages. PT lines isolated by Smith (1984) were used within

ten passages of cloning.

2.3.2. Freezing and thawing of cells

Frozen stocks were prepared by suspending cells in CM supplemented with 10% (v/v) DMSO. Aliquots (0.5–1.0ml) of this cell suspension were transferred to serum test tubes and stored overnight in racks in a −70°C freezer, such that they were cooled at a rate of about 1°C per minute. The tubes of cells were then transferred to the vapour above liquid nitrogen. In contrast to the freezing process, cells were thawed rapidly by placing the ampoules direcly into a 37°C water bath. The contents were transferred to a universal tube containing prewarmed medium. The DMSO was removed completely by spinning down the cells and resuspending in fresh medium before inoculating the culture vessel.

2.3.3. Preparation of feeder layers

Feeder layers were prepared from STO or 3T3 fibroblasts unless otherwise indicated. Confluent cultures were treated with medium containing 10µg/ml mitomycin-C for two hours at 37°C (Martin and Evans, 1975b). The cells were washed three times with PBSA, trypsinised and seeded out at a density of $4x10^4$cells/cm^2. The integrity of feeder layers depends critically on the condition of the cells at the time of mitomycin treatment. For the formation of good feeder layers it is important to use cells which are subject to density-dependent growth inhibition. High passage stocks (above passage 25), and stocks which had been overgrown with cells which continued to divide even at confluence, were discarded. Feeder layers were generally considered to be suitable for use for approximately one week after mitomycin treatment.

For ouabain rescue experiments (Section 2.11.) STO feeder layers were prepared *in situ*. Cultures were established at a density of $4x10^4/cm^2$ and exposed to mitomycin-C as above. The monolayers were washed three times with PBSA and incubated in CM for three hours. EC cells were then inoculated in fresh medium. Don and Don 6 feeder layers were likewise prepared *in situ*.

2.3.4. Establishment of Clonal Lines

Clonal lines were established by dilution plating of single cell suspensions into multi-well plates. Only where a single colony grew up in a well, was this trypsinised and expanded into a cell line.

2.4. KARYOLOGY

Log-phase cultures were treated with 100µg/ml colchicine for 2 hours at 37°C. Cells were trypsinised, spun down at 400*g* for 5 minutes and resuspended gently in 0.075M KCl. After a 4 minute incubation at room temperature, the cells were spun down again. The pellet was resuspended in a small volume of the hypotonic KCl solution and cells fixed by dropwise addition of ice-cold methanol:glacial acetic acid (3:1 v/v). They were left on ice for at least 30 minutes, then twice spun down and resuspended in fresh fixative. Drop preparations were made onto clean microscope slides. The chromosomes were stained with Giemsa stain, and chromosomes in twenty apparently unbroken metaphase spreads counted.

2.5. ELECTRON MICROSCOPY

For ultrastructural analysis by electron microscopy cells were grown as monolayers on Permanox dishes (Lux). They were washed twice in PBSA and once in 0.1M sodium cacodylate. Monolayers were then fixed *in situ* with 3% (v/v) glutaraldehyde in 0.1M sodium cacodylate and subjected to routine processing.

Sections were examined and photographed using a JEM-100S Electron Microscope.

2.6. MUTAGENESIS

Near-confluent monolayers of cells were treated with 2.5×10^{-5}M N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) at 37°C for 2 hours. The cells were then washed twice with unmodified medium, and incubated for a further 24 hours. They were trypsinised to remove dead and dying cells and reseeded

into a culture vessel of the same surface area. The cells were grown up to confluence again before starting selection procedures.

2.7. PREPARATION OF CONDITIONED MEDIUM

Feeder-conditioned medium was made by incubating 10ml of CM per 10^6 feeder cells for three days at 37°C. BRL-conditioned medium was prepared by incubating 30ml of medium per confluent 175cm^2 flask of BRL cells. Medium was harvested every three days for up to three weeks. Before use, conditioned medium was filtered through a Millipore (Harrow, Middx.) membrane, pore size 0.2-0.22µm. Control, unmodified medium was stored for three days at 37°C prior to use.

2.8. ANALYSES OF CELL GROWTH

Cells were plated at $5x10^4$ per 60mm dish in BRL-conditioned medium. After overnight incubation (Day 1) triplicate cultures were trypsinized and initial cell numbers determined using a haemocytometer. Cultures were then fed with the medium under test. They were refed on Day 3 and daily thereafter. Triplicate dishes were counted at 24 hour intervals for 7 days. Numbers of EC cells growing on feeders were calculated by subtracting the number of cells recovered from feeder layers alone.

2.9. PLATING TESTS

Cells were plated at 10^3 per 60mm culture dish by dilution of single cell suspensions. After overnight incubation the substance under test (or solvent alone for controls) was added. In experiments involving conditioned medium, dishes were refed three days later. Six days after addition of test substance, cultures were fixed and stained with Leishmans' stain (1.5g/l in methanol) for colony counting. A colony is defined as a cluster of four or more cells.

2.10. INDUCTION AND ANALYSIS OF EC CELL DIFFERENTIATION

2.10.1. Induction of Differentiation by Exposure to Retinoic Acid

Cells were seeded at a density of $4x10^3$ per cm^2 on gelatin and incubated overnight. Culture medium was then replaced with fresh medium supplemented with retinoic acid, added from a 1000-fold concentrate in DMSO (stored at -20°C). Medium in control dishes was supplemented with 0.1% (v/v) DMSO (0.012M). Cultures were refed every 3-4 days for up to 2 weeks.

2.10.2. Induction of Differentiation of B2B2, PSA4 and PSA4 Derivatives by Plating on Gelatin

Cells were seeded at a density of $4x10^3$ per cm^2 on gelatin in CM and incubated for up to eight days, feeding as necessary.

For monitoring purification of Differentiation Inhibiting Activity (below, Section 2.16) $2x10^4$ PSA4 cells, previously passaged on gelatin in BRL-conditioned medium, were inoculated into 16mm Linbro wells. After being allowed to attach for 4 hours, 0.1ml samples of the fractions under test were added to a final culture volume of 1.0ml. Cultures were maintained for 4 days and scored on the basis of morphology under phase contrast microscopy.

2.10.3. Induction of Differentiation by Formation of Aggregates

Cells were seeded at high density (>$2.5x10^6$ cells per 60mm dish) in Eagles medium supplemented with 10% foetal bovine serum but without non-essential amino acids, pyruvate or 2-mercaptoethanol (EfC10 medium). Cultures were fed as necessary until nests of cells were formed (Stage 1). Aggregates were detached by blowing fresh medium across the surface of the dish, transferred to bacteriological dishes to which they could not attach, and grown in suspension for 6 days in EfC10 medium (Stage 2).

After suspension culture, embryoid bodies were transfered to universal containers and allowed to sediment under gravity. They were fixed in Bouin's fluid or in 96% ethanol:glacial acetic acid (99:1 v/v) at 4°C (Dziadek and

Adamson, 1978), then spun down in molten bacteriological agar. The agar was allowed to set, and the embryoid bodies were subjected to routine histological processing and embedded in paraffin wax. 4-5µm sections were cut and then stained with Mayer's haemotoxylin and eosin.

2.10.4. Differentiation of Embryoid Body Outgrowths

Outgrowths were formed by allowing six day embryoid bodies to attach to Thermanox coverslips (Lux) in complete medium. The cultures were incubated for three days before refeeding. They were then refed at intervals for two weeks, fixed in 96% ethanol:glacial acetic acid (99:1 v/v), embedded in paraffin wax *in situ* , and after routine histological processing, sectioned, and stained with Mayer's haemotoxylin and eosin.

2.10.5. Fibrin Overlay Assay for Plasminogen Activator Production

A modification of the fibrin lysis method of Strickland and Mahdavi (1978) was used to assay colonies for enzyme activity. Cultures, established as for plating tests (Section 2.9), were incubated for 5 days, then washed twice with PBSA and overlaid with 1.5ml of serum-free medium containing 3.3mg/ml bovine fibrinogen, 0.4 units/ml human plasminogen (freed of low molecular weight material by gel-filtration on Sephadex G25M prior to use), and 0.67 units/ml bovine thrombin. Plasminogen was omitted from control dishes. After the formation of a fibrin gel, dishes were incubated at 37°C for 3-5 hours. The preparations were then fixed by placing upright in a closed container saturated with methanol vapour. They were stained with Kenacid blue R (2mg/ml in methanol: glacial acetic acid: water; 45:10:45; v/v), and examined for the presence of a lysis zone around each colony.

2.11. ESTIMATION OF METABOLIC COOPERATION BY RESCUE FROM OUABAIN TOXICITY

Cells were seeded at 10^3 per 60mm culture dish on STO feeders or on gelatin. In the standard procedure (Hooper and Morgan, 1979), after overnight incubation half the dishes were treated with medium containing 3mM ouabain and half with unmodified medium. Three days later, all the dishes were

returned to normal medium for 3-4 more days growth. Dishes were then fixed and stained with Leishmans' for colony counting.

In a modified procedure for testing the effects of retinoic acid on cooperation, the desired concentration of the retinoid was added to the cultures 24 hours prior to addition of ouabain and was maintained throughout the incubation with ouabain.

The index of cooperation is defined as the mean number of colonies which grow up in the presence of ouabain, divided by the mean number in its absence, divided again by the corresponding ratio for a control population. A high ratio corresponds to rescue of a large proportion of the EC cells by the mechanism discussed in Section 1.8., and a low ratio to poor rescue. Cells cooperating as well as the control should give a ratio of 1.

2.12. DETECTION AND ESTIMATION OF CELLULAR RETINOIC ACID BINDING PROTEIN (CRABP)

CRABP was assayed using a modified version of the sucrose gradient procedure developed by Ong and Chytil (1975). Exponentially growing cultures were trypsinized and the trypsin then neutralized with serum-containing medium. The cells were washed three times by centrifugation in PBSA. They were lysed with 20 strokes of a hand homogeniser at a density of approximately 10^8 per ml in 10mM Tris.HCl/7mM 2-mercaptoethanol/2mM $MgCl_2$, pH 7.4, containing 0.1mg/ml α_2-macroglobulin. Cytosol supernatants were obtained by centrifugation at 100,000*g* and 4°C for 1 hour. Aliquots (0.3ml) of cytosol were incubated for 4 hours in the dark at 4°C with 30nM [^{3}H]-retinoic acid. Parallel incubations were carried out with a 200-fold excess of unlabelled all-*trans*-retinoic acid. Retinoids were added in ethanol (final concentration, 0.33% v/v). In experiments involving retinol or retinal, the ethanol was supplemented with the antioxidant butylated hydroxytoluene (1mg/ml).

Prior to density gradient centrifugation, 0.2ml of charcoal/dextran suspension (2mg/ml in PBSA) was added to each sample to adsorb unbound ligand (Korenman, 1968). After 5 min incubation, over 90% of free retinoid was removed by centrifugation at 8,000*g*. Aliquots (0.2ml) of the supernatant were applied to 4-16% (w/v) sucrose gradients in PBSA. Linear gradients were

prepared by upward displacement using an MSE mini-gradient former. Centrifugation was performed at 200,000*g* and 4°C for 18 hours in a Beckman SW60Ti swing-out rotor using a Sorvall OTD-50 ultracentrifuge. Fractions (0.15ml) were collected and radioactivity quantified by liquid scintillation counting.

CRABP activity was estimated in terms of pmol [^{3}H]-retinoic acid bound/mg cytosol protein by determining the counts specifically bound in the 2S region of the gradient. For routine detection of CRABP a simplified procedure (adapted from Sherman *et al*, 1983b) was employed which utilises the fact that these cytosols contain only 2S retinoic acid binding activity and are devoid of contaminating serum binding proteins. Sucrose gradient centrifugation was omitted and radioactivity measured in the supernatants directly after extraction of unbound retinoid with charcoal/dextran. This method gave qualitatively identical results to the sucrose gradient assay, but was not quantitatively reproducible.

Freshly prepared cytosols were used for quantitative determinations, as binding activity was often found to decline on frozen storage, in contrast to the finding of Schindler *et al* (1981).

Nuclear fractions were prepared according to the method of Jetten and Jetten (1979). Cell lysates were centrifuged at 1000*g* for 5 min. The pellet was resuspended in 0.032M sucrose solution containing 3mM $MgCl_2$, layered on 1.7M sucrose and centrifuged at 86,000*g* and 4°C for 1 hour in a SW40Ti swing-out rotor. Intact nuclei in the pellet were homogenised by sonication and centrifuged at 100,000*g* for 1 hour. The resulting supernatant is the nuclear fraction.

2.13. RETINOIC ACID UPTAKE ASSAY

Measurement of the association of retinoic acid with EC cells was performed in Leighton tubes (Nunc). Cells were inoculated at $5x10^5$ per tube and cultured for 44 hours in feeder-conditioned medium until near-confluent. Tubes were then placed upright in a 37°C water bath. Blank tubes were included in the assay to control for adsorption of retinoic acid to the plastic. Culture medium was aspirated and monolayers gently washed twice with warm PBSA. Serum-free medium supplemented with BSA (1.5mg/ml) and containing 8nM

[^{3}H]-retinoic acid plus various concentrations of unlabelled retinoic acid was added to each tube.

Uptake was halted by aspirating off radioactive medium, placing the tubes on ice immediately and washing three times with ice-cold PBSA. Cells were lysed by incubation with Folin's A (Na_2CO_3, 2% w/v, in 0.1M NaOH) for 2 hours. Lysates were neutralized with 4M HCl and transferred to scintillation vials for counting.

Cell numbers were determined by harvesting of triplicate cultures grown in parallel with the test cultures and subjected to the same washing procedures.

2.14. PREPARATION OF PLASMA MEMBRANE FRACTIONS

Plasma membranes were prepared by the hypo-osmotic lysis method of Thom *et al* (1977) from mass microcarrier cultures of EC cells grown on 3T3 feeders. Cells were harvested by trypsinization, washed twice by centrifugation in PBSA and stored as a frozen pellet at -20°C. Subsequently cells were thawed, washed by centrifugation in 0.05M boric acid, 0.15M NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, pH 7.2, and the pellet resuspended in 2 vol of this solution. Succeeding steps were performed at 4°C.

The cell suspension was added slowly with constant stirring to 100 vol of 0.02M boric acid, 0.2mM EDTA, pH 10.2. After stirring for 10 min, 8 vol of 0.5M boric acid, pH 10.2 were added and stirring continued for a further 5 min. The lysate was filtered through medical gauze and centrifuged at 450*g* for 10 min. The supernatant was recentrifuged at 12,000*g* for 30 min. Pelleted material was resuspended in complete phosphate-buffered saline (PBS) i.e. PBSA plus 132.5mg/l $CaCl_2$ and 100mg/l $MgCl_2$, and layered on top of 35% (w/w) sucrose in complete PBS. Centifugation was carried out at 24,000*g* for 1 hour in a Beckman SW41Ti swing-out rotor. Material at the interface was collected using siliconized Pasteur pipettes. The membranes were washed by centrifugation in complete PBS at 100,000*g* and stored at -70°C until needed.

Samples for electron microscopy were fixed in 3% (v/v) glutaraldehyde in 0.1M sodium cacodylate, pH 7.4, for 4 hours at room temperature. They were washed overnight in 0.1M sodium cacodylate/0.25M sucrose, then post-fixed in 1% osmic acid in Michaelis buffer (0.143M sodium barbitone/0.143M sodium

acetate) at pH 7.4 and 0°C for 2 hours. Samples were stained with 1% uranyl acetate for 2 hours at room temperature and routinely processed.

2.15. ELECTRON SPIN RESONANCE

Plasma membrane preparations were pooled, pelleted and resuspended in complete PBS at a concentration of approximately 10^{10} cell equivalents per ml. The nitroxide spin-label 5-(4',4'-dimethyloxazolidine-N-oxyl)stearate (5-doxyl stearate) was added to a concentration of 130µg/ml from a 4mg/ml stock solution in ethanol. Spectra were resolved on a Varian ESR spectrometer fitted with a heater and thermocouple in the Department of Biochemistry, Edinburgh University. A modulation amplitude of 3.2×10^{3}G and microwave power of 20mW were employed to obtain spectra.

Approximate order parameters, S_{app}, were calculated from the line splittings according to the equation:-

$$S_{app} = \frac{A_{max} - A_{min}}{A_{zz} - 1/2(A_{xx} + A_{yy})}$$

where A_{max} is the maximum hyperfine splitting, A_{min} is the minimum hyperfine splitting and A_{zz}, A_{xx}, A_{yy} are the single crystal, principle hyperfine splittings for the doxyl group (see Appendix).

2.16. PARTIAL PURIFICATION OF DIFFERENTIATION INHIBITING ACTIVITY (DIA)

Initial stages were based on the method of Marquardt *et al* (1981) for isolation of rat IGF-II (MSA) from BRL-conditioned medium. Cells were grown to confluence on 2.5g of microcarriers in 1l of CM, then washed with serum-free medium. They were incubated overnight in serum-free medium which was discarded. Conditioned serum-free medium was then collected in 900ml batches every 4-5 days for two weeks. It was clarified by centrifugation at 27,500*g* and 4°C for 1 hour and stored at -20°C in the presence of α_2-macroglobulin (1µg/ml).

All subsequent steps were performed at 4°C. Pooled samples of conditioned medium were buffered by addition of solid Hepes to a final concentration of

40mM and concentrated 10-12 fold using immersible CX-10 ultrafilters (molecular weight limit, 10,000)(Millipore). The concentrate was dialysed in SpectraPor 6 dialysis tubing (molecular weight cut-off, 2000)(Pierce and Warriner, Chester, Cheshire) for 60 hours against 5 changes of 0.1M acetic acid, then centrifuged at 100,000 *g* for 45 min. The supernatant was lyophilized and reconstituted in a small volume of 0.67M acetic acid. This fraction is hereafter referred to as the Dialysate. It was applied to a 2.5 x 80cm^2 column of Bio-Gel P-10 pre-equilibrated with 0.67M acetic acid. Flow rate was 13ml/hour and 6.5ml fractions were collected. Protein elution was monitored by absorption at 280nm using an LKB Uvicord II photometer.

Aliquots (0.2ml) were lyophilized, dissolved in 25µl 0.1M acetic acid and made up to 0.5ml with MSA buffer (serum-free medium containing 1mg/ml bovine serum albumin and 50mM 2-[bis(2-hydroxyethyl)amino]ethanesulphonic acid, pH7.0). These samples were assayed for capacity to inhibit differentiation of PSA4 on gelatin (Section 2.10.2.) and for mitogenic activity on rat kidney fibroblasts (Section 2.17).

Fractions containing Differentiation Inhibiting Activity (DIA) were pooled, lyophilized to dryness and reconstituted in a small volume of 0.67M acetic acid. This fraction is hereafter referred to as the P-10 Fraction. It was applied to a Sephadex G-75 column (2.5x90cm^2), flow rate 12ml/hour, calibrated with the Sigma GF-70 molecular weight marker kit. Fractions (3ml) were collected and assayed as for the P-10 eluant. Selected samples containg DIA and/or mitogenic activity were analysed by SDS-PAGE (Section 2.18.). Those samples containing DIA were then pooled, lyophilized to dryness, and reconstituted in 0.1 vol of 0.1M acetic acid. This sample is termed the G-75 Fraction.

2.17. GROWTH-PROMOTING ASSAY ON NRK 49F CELLS

Mitogenic activity was quantified as the stimulation of [^{3}H]-thymidine incorporation by quiescent rat kidney fibroblasts (Marquardt *et al*, 1980). NRK 49F cells were seeded at 2x10^4/well in CM into 16mm Linbro wells containing Thermanox coverslips. After 24 hours CM was replaced by culture medium containing only 0.1% foetal bovine serum (1.0ml/well). Five days later the sample to be tested was added in a total volume of 0.1ml of MSA buffer. Cultures were incubated for 16 hours, then exposed for 5 hours to 4µCi of

[^{3}H]-thymidine plus a 10-fold excess of unlabelled thymidine. The monolayers were washed three times with PBSA and fixed by incubation with ice-cold methanol/acetic acid (3:1, v/v) for 15 min. The coverslips were then incubated in 10% trichloracetic acid for 1 hour at 4°C to extract unincorporated nucleotides. They were washed twice in 10% trichloroacetic acid and once in 70% ethanol, before transfer to scintillation vials and counting of radioactivity.

2.18. DISCONTINUOUS SODIUM DODECYL SULPHATE-POYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Analytical SDS-PAGE was performed by a modification of the discontinuous buffer system of Laemmli (1970). Samples (10-15µg protein) were lyophilized to dryness and reconstituted in sample buffer; 125mm Tris.HCl, pH7.0/10mm EDTA/4% (w/v) SDS/40% (v/v) glycerol. For reduced samples 10% (v/v) 2-mercaptoethanol was included in the buffer. Both non-reduced and reduced samples were boiled for 5 min then cooled on ice immediately prior to loading the gel. Electrophoresis was conducted at 30mA for 4-5 hours in an LKB Vertical Electrophoresis Unit using a 15% separating gel and a 5% stacking gel.

Proteins were visualized by staining with Kenacid blue (2mg/ml in methanol: acetic acid: water; 45:10:45) and/or with ammoniacal silver (Oakley *et al*, 1980). Gels were calibrated with the Sigma SDS-70L molecular weight marker kit.

2.19. INDIRECT IMMUNOPEROXIDASE

EC cells were cultured on gelatinised 22mm Thermanox coverslips for 4-5 days in the medium under test. The monolayers were washed three times with PBSA and fixed by immersion in acetone for 5 min. The air-dried preparations were mounted, cell side uppermost, on microscope slides. They were incubated for 10 min with normal rabbit serum, diluted 1:5 (v/v) with 12.5M tris buffered saline, pH7.6 (NRS/TBS). Monoclonal antibody was then applied to the test sample for 30 min. Antibodies 2H9 and M1/22-25 were in the form of culture supernatants and were used undiluted. Anti-SSEA-1 was an ascites fluid and was diluted 1:10 with NRS/TBS before use. Duplicate specimens were incubated with NRS/TBS alone. The samples were washed thoroughly with TBS and incubated for a further 10 min with NRS/TBS. Peroxidase-conjugated rabbit anti-mouse immunoglobulin, diluted 1:20 with NRS/TBS was applied to

all specimens for 30 min. After thorough washing in TBS, the slides were incubated with diaminobenzidine (1mg/ml in 0.5M tris/HCl, pH7.6) for 5–10 min. The reaction was quenched by immersion in water and specimens counterstained with haematoxylin.

2.20. DETERMINATION OF PROTEIN

Protein was determined by the method of Lowry *et al* (1951) or by a standard dye-binding assay based on the change in light absorption of Coomassie Brilliant Blue G-250 in response to protein concentration variation (Bradford, 1976). Several dilutions of bovine serum albumin solution were employed as standards.

CHAPTER 3

STUDIES ON PTMR0: AN EMBRYONAL CARCINOMA CELL LINE RESISTANT TO THE INHIBITION OF METABOLIC COOPERATION BY RETINOIC ACID

3.1. INTRODUCTION

PTmr0 is a pluripotent embryonal carcinoma cell line resistant to the inhibition of junctional communication by retinoic acid (Smith, 1984; Smith *et al*, submitted). It is a variant of PSA4TG12 selected for the ability to cooperate in the presence of 10^{-4}M retinoic acid by the ouabain "kiss of life" procedure (Section 1.8.). Unlike all other EC and non-EC cells tested to date (Smith, 1984; Pitts *et al*, in press), PTmr0 cooperates normally in high concentrations of retinoic acid.

In addition to blocking junctional transfer, retinoic acid has a variety of other actions on EC cells, including the induction of differentiation (Section 1.5) The availability of PTmr0, with its altered response of junctional communication, provides one means of investigating the relationship of this effect to the other actions of retinoic acid. Wild type responses to retinoic acid retained by PTmr0 are unlikely to be mediated via, or otherwise connected with, the inhibition of metabolic cooperation.

PTmr0 is the first variant of its type and elucidation of the biochemical nature of the lesion could shed light on the mode of action of retinoic acid not only on junctional communication but possibly also on other cellular functions.

Section 3.2 is an extension of the work of Smith (1984) on characterisation of some of the biological responses of PTmr0 and its parent line PSA4TG12 to retinoic acid. The work described in Section 3.3 is a comparison of some of the biochemical properties of the two cell lines.

3.2. CELL BIOLOGY

3.2.1. Retinoic Acid-Induced Differentiation

Retinoic acid has been found to induce differentiation of all feeder-independent murine EC cell lines tested (Strickland and Mahdavi, 1978; Jetten *et al*, 1979) with the exception of specifically selected differentiation-defective (dif^-) variants (Section 1.5.). However, the effects of retinoic acid on differentiation of feeder-dependent EC monolayers are poorly characterised. This is because differentiation is inhibited in the presence of a feeder-layer (Ogiso *et al*, 1982; Smith and Hooper, 1983) whilst in the absence of feeders these cells die or differentiate (Section 1.4.).

PTmr0 is pluripotent, differentiating into a variety of cell and tissue types following aggregation (Smith, 1984; Smith *et al*, submitted). Retinoic acid has ill-defined effects on this system, but there are no obvious differences in the responses of PTmr0 and PSA4TG12.

Like PSA4TG12, PTmr0 undergoes differentiation when plated in the absence of feeders on gelatinised culture surfaces in medium containing 2-mercaptoethanol (Plate 3.1A). The effects of retinoic acid on differentiation in such circumstances appear intractable to conventional analysis: not only is there the problem of resolving retinoic acid-induced from spontaneous differentiation, but the system is complicated by the toxic actions of retinoic acid which greatly increase an already considerable level of cell death (see below Section 3.2.2.)

However, these difficulties can be circumvented by the use of medium conditioned by Buffalo rat liver cells. This medium enables feeder-dependent EC cells to be maintained as near pure stem cell populations in the absence of feeder layers, even at cloning density (see Chapter 5.). PSA4TG12 and PTmr0 show very low levels of spontaneous differentiation when cultured on gelatinised substrata in 60% BRL-conditioned medium : 40% CM, henceforth referred to as BRL-medium (see also Section 5.3). When examined by light microscopy, less than 5% of colonies contain morphologically differentiated cells (Plate 3.2A,B). The addition of retinoic acid to BRL-medium induces differentiation of feeder-dependent EC cells in a concentration-dependent

manner (see Chapter 5) as occurs with feeder-independent cells in CM (Strickland and Mahdavi, 1978). PTmr0 cells cultured in 10^{-6}M retinoic acid for 6 days are transformed into monolayers apparently consisting wholly of differentiated cells (Plate 3.2C,D). These cultures consist predominantly of refractile epithelioid cells, but also contain some fibroblasts. They are distinct from the pavements of flattened cells which predominate on spontaneous differentiation in CM containing foetal calf serum (Plate 3.1; see also Section 5.3.6). Residual EC cells are not detectable by microscopic examination, but some probably persist, as a few EC-like colonies generally grow up if the retinoic acid is removed.

At 10^{-7}M retinoic acid residual clumps of EC cells can be identified under the light microscope. These are usually in close association with differentiated cells and only rarely appear as discrete EC colonies. At lower concentrations both the total proportion of EC cells and the numbers of pure EC colonies increase. At 10^{-10}M retinoic acid there are no discernible differences from control populations. The concentration of retinoic acid does not appear to affect the types of differentiated cells formed, nor greatly to alter their relative proportions.

This pattern of response to retinoic acid in BRL-medium is similar to that shown by PSA4TG12 and also by the EK line B2B2 and the parental PSA4 EC line (Section 5.3.6.). The only difference in the response of PTmr0 from that of these other cell lines is that differentiation of the former appears less extensive at equivalent concentrations of retinoic acid over the 10^{-9}-10^{-7}M range.

Confirmation of slight differences in sensitivity detected by light microscopy requires quantification of some index of differentiation. Plasminogen-dependent fibrinolytic assays for plasminogen activator were therefore employed to obtain a quantitative estimate of the differentiation responses of PTmr0 and PSA4TG12 to different concentrations of retinoic acid. The enzyme plasminogen activator is not produced by EC cells but is produced by many other embryonic cell types (Bode and Dziadek, 1979; Marotti *et al*, 1982). It has been widely used as a measure of EC cell differentiation (Strickland and Mahdavi, 1978; Jetten *et al*, 1979; Lo and Gilula, 1980a; Rizzino, 1983a,b; Sherman *et al*, 1983; Smith and Hooper, 1983). The fibrin overlay assay detects plasminogen activator production by individual EC colonies. Under the conditions used here (Section 2.10.5.) most, if not all, of these colonies are derived from single cells. The

Plate 3.1 Differentiation of PTmr0 Induced by Plating in the Absence of Feeders in CM or by Exposure to Retinoic Acid in BRL-Medium

Phase contrast

	Magnification
A. PTmr0 colony 6 days after plating in CM	106
B. PTmr0 colony 6 days after plating in BRL-medium plus 10^{-7}M retinoic acid	106

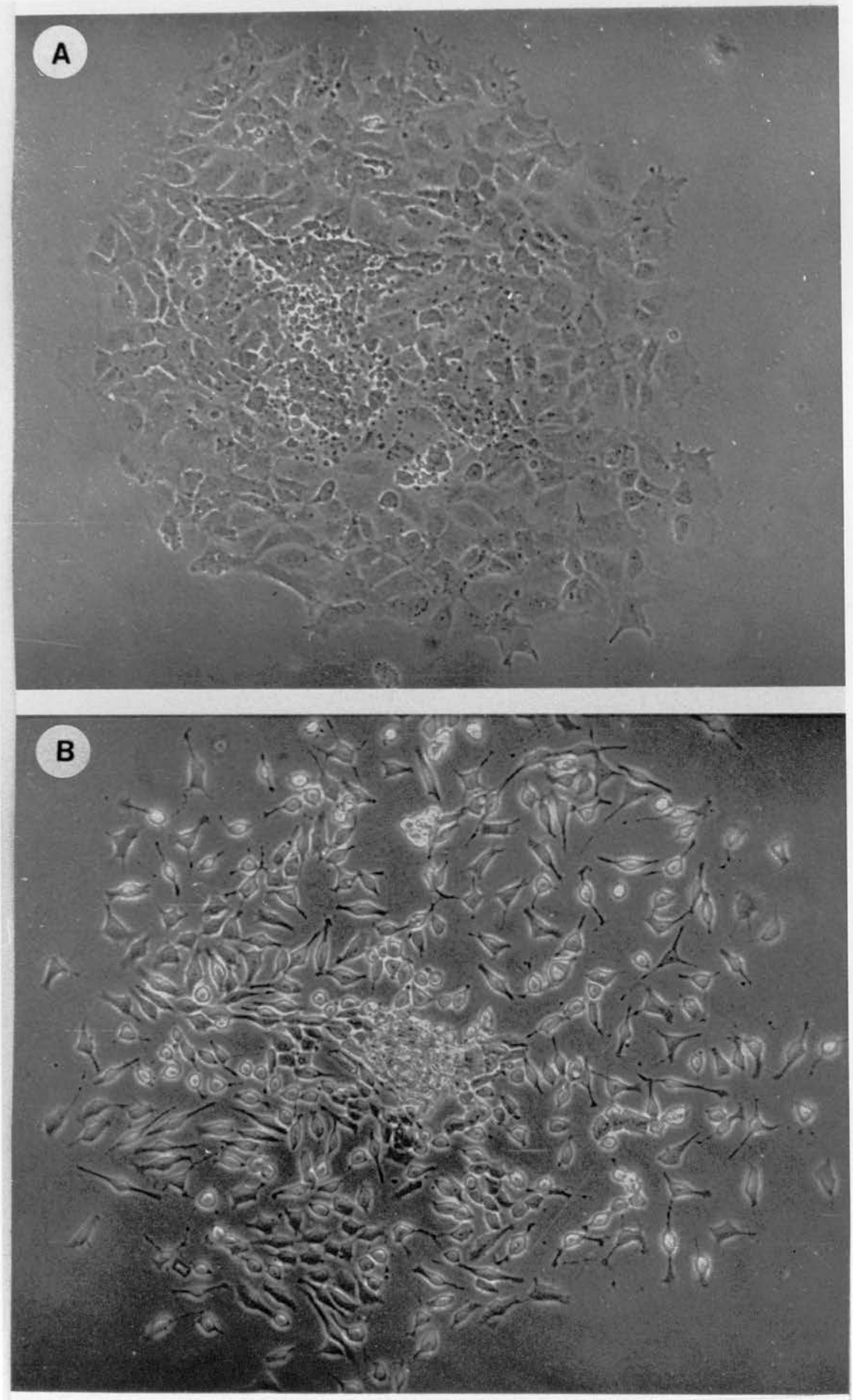
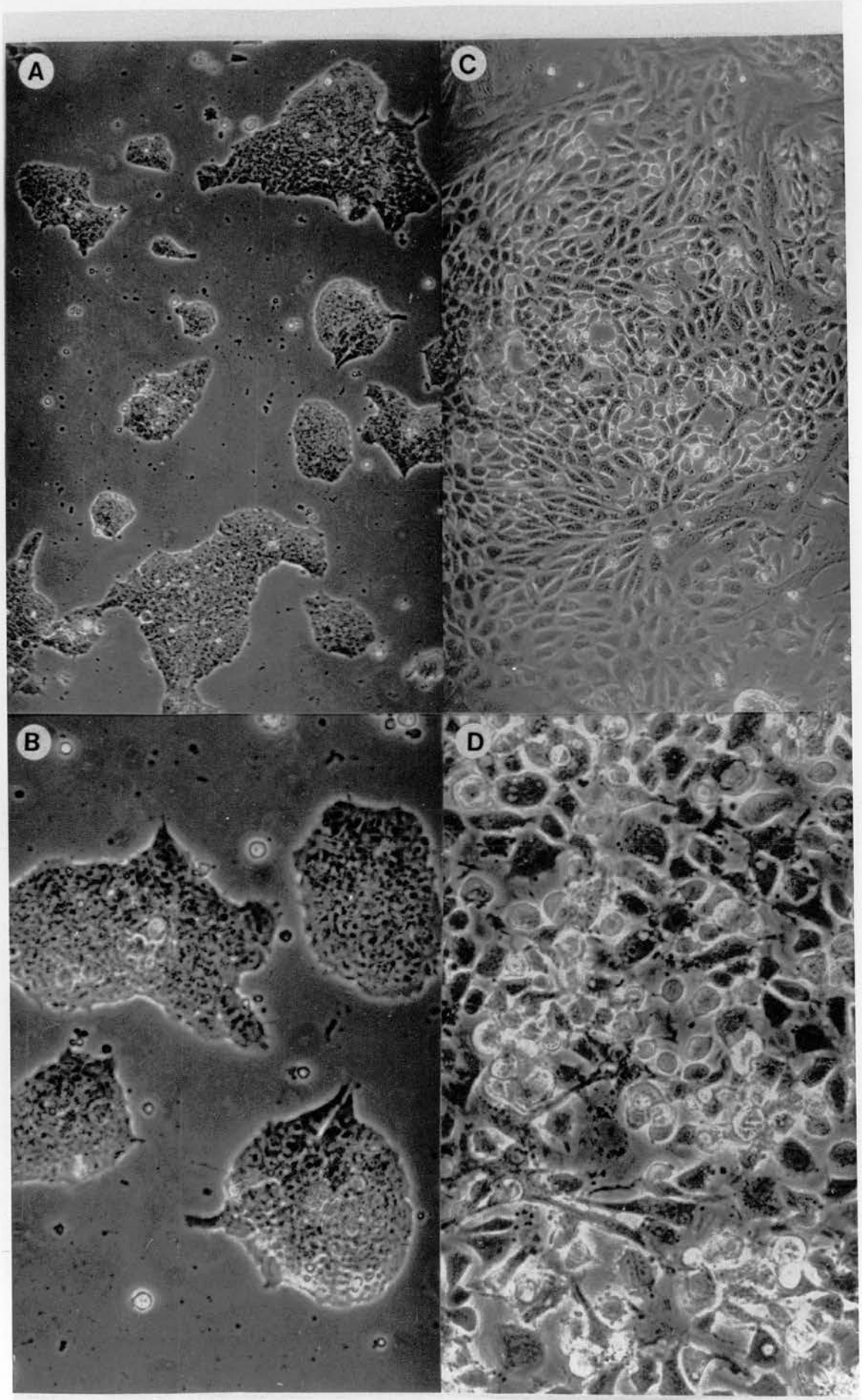

A
B

Plate 3.2 Retinoic Acid-Induced Differentiation of PTmr0

PTmr0 cells were passaged twice in BRL-medium in the absence of feeders and seeded at $4x10^3/cm^2$ into gelatinised flasks in BRL-medium in the presence or absence of $10^{-7}M$ retinoic acid. Phase contrast optics were employed.

	Magnification
A. BRL-medium, 6 days	124
B. BRL-medium, 6 days	360
C. BRL-medium plus retinoic acid, 6 days	90
D. BRL-medium plus retinoic acid, 6 days, followed by 5 days in BRL-medium without retinoic acid	360


A
C
B
D

assay therefore provides a measure of the proportion of stem cells yielding differentiated derivatives under given conditions.

Table 3.1 contains the results of one experiment on the effects of 10^{-7}M retinoic acid. It is apparent that under appropriate conditions essentially all surviving PSA4TG12 or PTmr0 colonies produce lytic zones. No lysis occurred in controls in which plasminogen was omitted from the overlay. As the toxic effects of retinoic acid on PTmr0 are not very pronounced at 10^{-7}M in BRL-medium (see following Section) it may be concluded that this cell line does not contain a significant subpopulation resistant to induction of differentiation by retinoic acid.

Table 3.2 details the concentrations of retinoic acid inducing plasminogen activator production by 50% of colonies in two independent experiments. They appear to confirm the indications from observations on mass cultures described above that around 10-fold greater concentrations of retinoic acid are required to induce the same degree of differentiation in PTmr0 as is obtained with PSA4TG12.

Table 3.1
Fibrin Overlay Assay for Plasminogen Activator Production by PTmr0 and PSA4TG12 in Response to 10^{-7}M Retinoic Acid

Cell Line	Treatment	Number of Colonies	Number of Lytic Zones	% Colonies Producing Plasminogen Activator
PSA4TG12	Control	252	14	5.5
"	"	270	4	1.5
"	RA	166	162	97.6
"	"	214	186	86.9
PTmr0	Control	150	0	0.0
"	"	138	8	5.8
"	RA	90	86	95.6
"	"	108	104	96.2

Each row of figures refers to a single dish

Table 3.2
Concentrations of Retinoic Acid Inducing Lysis by 50% of PSA4TG12 and PTmr0 Colonies in Fibrin Overlay Assays

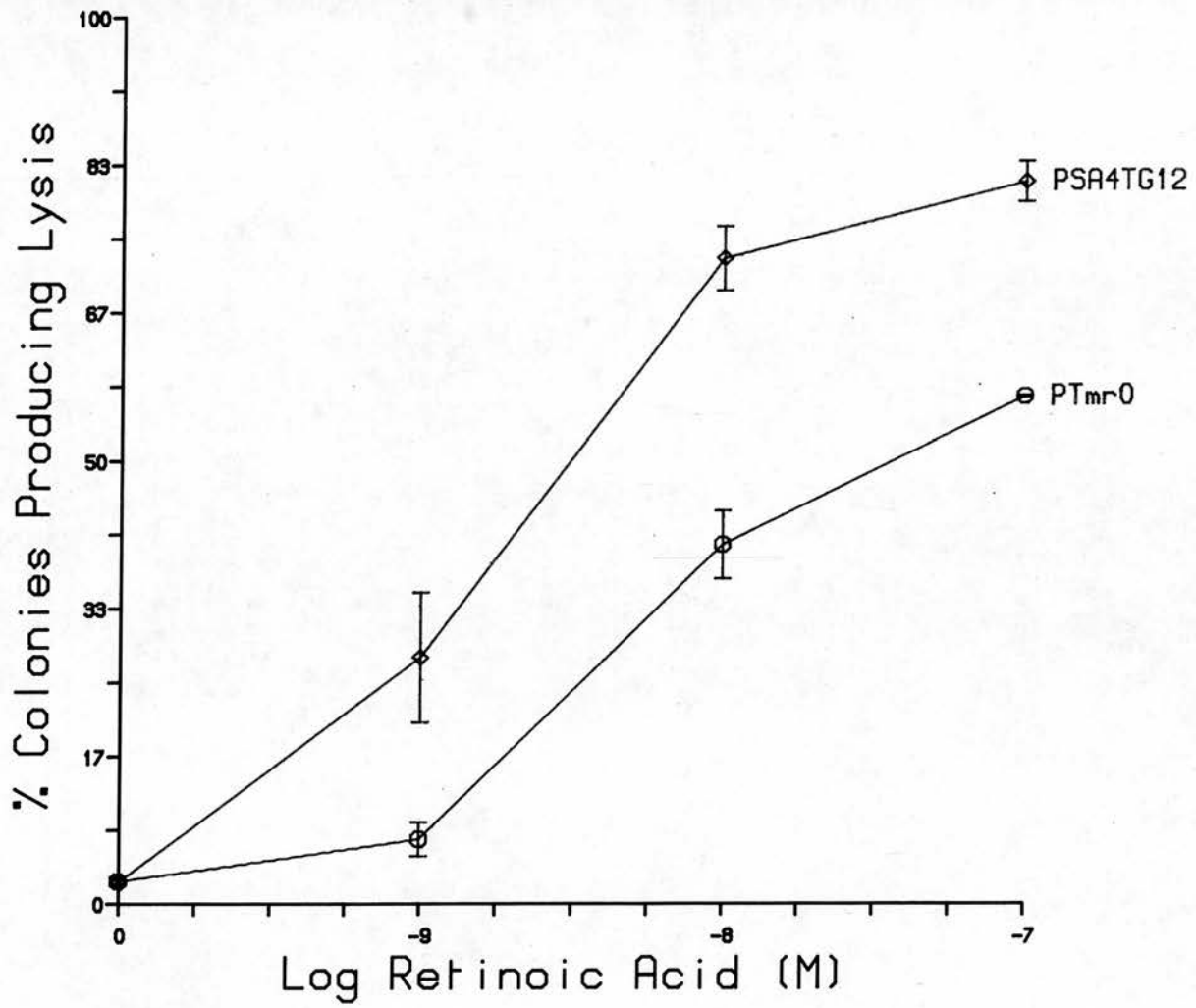
Cell Line	Expt.1	Expt.2	Mean
PSA4TG12	1.6×10^{-9}M	5.0×10^{-9}M	3.3×10^{-9}M
PTmr0	5.0×10^{-8}M	1.8×10^{-8}M	3.4×10^{-8}M

Values determined from dose response curves over the 10^{-9}-10^{-7}M concentration range with duplicate determinations at each concentration (Fig.3.1).

Fig.3.1 Induction of Plasminogen Activator by Retinoic Acid

Fibrin overlay assays were performed in duplicate as described in Section 2.10.5. on cultures of PSA4TG12 (blue line) and PTmr0 (red line) in BRL-medium. Each data point is the mean±1/2 range determined from two independent experiments.

Fig.3.1 Induction of Plasminogen Activator by Retinoic Acid



3.2.2. Retinoic Acid Toxicity

In addition to its effects on differentiation, retinoic acid reduces the growth rate of EC cells (Schindler *et al*, 1981; Rosenstraus *et al*, 1982) and induces cell death (Strickland and Mahdavi, 1978; Rayner and Graham, 1982; Mummery *et al*, 1984; Rayner and Pulsford, 1984). Reduction in the rate of cell proliferation does not arise by selection of a slow-growing subpopulation (Rayner and Graham, 1982). It commences after the onset of differentiation (Mummery *et al*, 1984) and is, at least in part, a consequence thereof (Schindler *et al*, 1981; Rayner and Graham, 1982; Rayner and Pulsford, 1984). Toxicity in contrast may be manifest in the undifferentiated EC population and there is some evidence that it could be associated with the acquisition of the commitment to differentiate (Mummery *et al*, 1984; see below Sections 4.5.3., 4.5.4. and 4.6).

EC cell lines differ in their susceptibility to retinoic acid toxicity (Strickland and Mahdavi, 1978). The selection of PTmr0 entailed culture in highly toxic concentrations of retinoic acid (10^{-4}M) (although in the presence of STO feeders). It was therefore considered possible that the variant might show an altered susceptibility to retinoic acid toxicity which might in turn be related to its altered junctional communication properties.

Toxicity to EC cells cannot be assayed by determination of cell numbers after exposure to retinoic acid because of its effects on differentiation and hence cell proliferation, as discussed above. Nor can it accurately be measured by plating tests in the presence of feeder cells, as it is difficult to distinguish differentiated teratocarcinoma cells from the feeder layer background. Moreover, feeder cells may facilitate cell survival in otherwise toxic conditions. Retinoic acid toxicity to PTmr0 and PSA4TG12 was therefore assessed by plating tests on gelatinised substrata in either CM or BRL-medium.

The results of toxicity tests in CM and in BRL-medium are illustrated in Fig.3.2. $L.D._{50}$ values determined from the individual dose-response curves are tabulated in Table 3.3.

Fig.3.2 Toxicity of Retinoic Acid to PTmr0 and PSA4TG12 in CM and BRL-Medium

Plating tests were performed as described in Section 2.9 on triplicate cultures. Each data point for CM (solid lines) is the mean±s.e.m. determined from three independent experiments. BRL-medium was employed in only two of these experiments, results for which (broken lines) are plotted as mean±1/2 range.

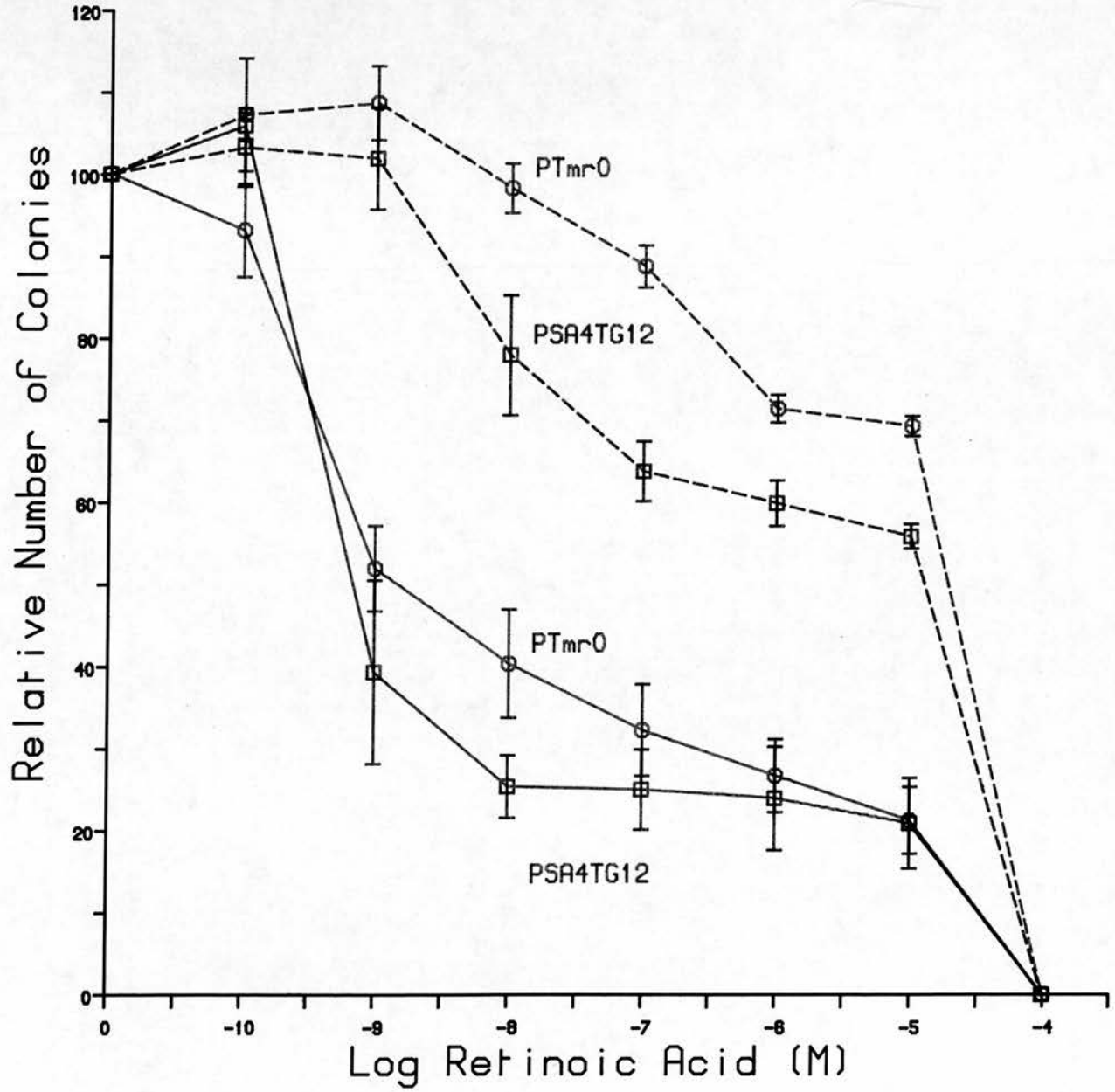

Relative Number of Colonies
120
100
80
60
40
20
0
0
-10
-9
-8
-7
-6
-5
-4
Log Retinoic Acid (M)
PTmr0
PSA4TG12
PTmr0
PSA4TG12

Table 3.3
L.D.$_{50}$ Values for Retinoic Acid Toxicity to PTmr0 and PSA4TG12 in CM and BRL-Medium

	PTmr0		PSA4TG12	
	CM	BRL-Medium	CM	BRL-Medium
Expt.1	5.6×10^{-9}M	-	$<10^{-9}$M	-
Expt.2	7.1×10^{-10}M	2.0×10^{-5}M	1.4×10^{-9}M	1.3×10^{-5}M
Expt.3	3.5×10^{-9}M	1.8×10^{-5}M	8.9×10^{-10}M	1.3×10^{-5}M
Mean	3.3×10^{-9}M	1.9×10^{-5}M	1.1×10^{-9}M*	1.3×10^{-5}M

*Excluding Expt.1

There is a suggestion that PTmr0 is slightly less susceptible to retinoic acid toxicity in CM than PSA4TG12, but the difference in L.D.$_{50}$ values is not statistically significant (*t* test, $P>0.05$). On prolonged exposure retinoic acid kills 50% of cells of both lines at concentrations at least 4 orders of magnitude lower than those at which it blocks metabolic co-operation in PSA4TG12 (see Section 3.4 for discussion).

Both cell lines are more resistant to retinoic acid in BRL-medium than in CM. L.D.$_{50}$ values for both PSA4TG12 and PTmr0 under these conditions are at the lower limit of the concentration range over which retinoic acid has an inhibitory effect on junctional communication in wild type cells. PTmr0 appears slightly more resistant than its parent line, though there is insufficient data to assess the statistical significance of such a conclusion. Number of colonies formed by PTmr0 as a percentage of cells seeded varied from 10.5-22.6% for control dishes in CM. This was increased by a factor of approximately 1.5 in BRL-medium. The number of colonies formed by PSA4TG12 was greater than that by PTmr0 by a factor of 1.6-2.2 in all experiments and was similarly enhanced in BRL-medium. There was no correlation between numbers of colonies formed in the absence of retinoic acid and L.D.$_{50}$ in individual experiments.

The higher concentration of DMSO required for dissolution of 10^{-4}M retinoic acid (0.04M compared with 0.012M) had no significant effect on cloning efficiency in control dishes in BRL-medium. Therefore the acute toxic effects observed at 10^{-4}M retinoic acid can be ascribed to the retinoid. This level of retinoic acid has been reported to be highly toxic to many cell types in culture and *in vivo* (Fell *et al*, 1962; Wang *et al*, 1975; Wiggert *et al*, 1977; Harrison *et al*, 1979, Lotan, 1980; Meeks *et al*, 1981; Qin *et al*, 1983)

Greater resistance in BRL-medium than in CM does not arise from inactivation or degradation of the inducer, as Pitts *et al* (in press) have shown that retinoic acid preincubated with BRL cells remains capable of blocking junctional communication and the data presented in Section 3.2.1. demonstrate that a differentiation response by PSA4TG12 is detectable at 10^{-9}M retinoic acid.

In CM considerable numbers of EC cells die in the absence of any added toxic agent, whereas cell death in BRL-medium is relatively low so long as neutral pH is maintained (Section 5.2.). This is underscored by the difference in the numbers of colonies formed in the two media. Colony formation by 30-60% of cells seeded, which is found for feeder-dependent EC cells in BRL-medium is comparable to the behaviour of feeder-independent lines such as PC13.5 in CM. Lower numbers of colonies obtained in CM may be due to poorer culture conditions and/or associated with the spontaneous differentiation of feeder-dependent EC lines which occurs in these conditions but is inhibited by BRL-medium (Section 5.3.).

The fact that PSA4TG12 and PTmr0 cells in CM spontaneously become committed to differentiate and that this is accompanied by a high level of cell death, may render these cells acutely susceptible to toxic agents and/or to agents which promote differentiation. EC cells proliferating as a stem cell population, in contrast, may be intrinsically more resistant. Rayner and Graham (1982) found that the extent of retinoic acid toxicity to the EC line PC13.5 was dependent on the culture environment. In addition, BRL-medium may contain factors which enhance cell viability or directly inhibit retinoid toxicity. It is also possible, however, that the reduction of cell mortality in BRL-medium could be related to its differentiation-inhibiting activity. Evidence in support of this idea will be presented in Chapters 4 and 5.

3.3. BIOCHEMICAL STUDIES

3.3.1. CRABP Analyses on Cytosols

Specific intracellular receptors have been proposed as mediators of retinoid action (reviewed by Chytil and Ong, 1978) following the paradigm of the steroid hormones (O'Malley and Means, 1974). Cellular retinoic acid binding protein (CRABP) has been implicated in the induction of EC cell differentiation by

retinoic acid (Jetten and Jetten, 1979; Schindler *et al*, 1981). Although the effect on junctional communication is too rapid to be mediated via *de novo* gene expression (Pitts *et al*, in press), it remains possible that CRABP could be involved via some other mechanism (see discussion in Section 1.5. on the modes of action of retinoids). The structural requirements for retinoid activity on metabolic cooperation (Pitts *et al*, 1985) show similarities to the requirements for binding to CRABP (Chytil and Ong, 1978; Jetten and Jetten, 1979). Thus retinol and retinol esters which do not bind to CRABP are inactive, and the terminal carboxyl group is necessary but not sufficient for both binding to CRABP and maximal inhibition of junctional communication.

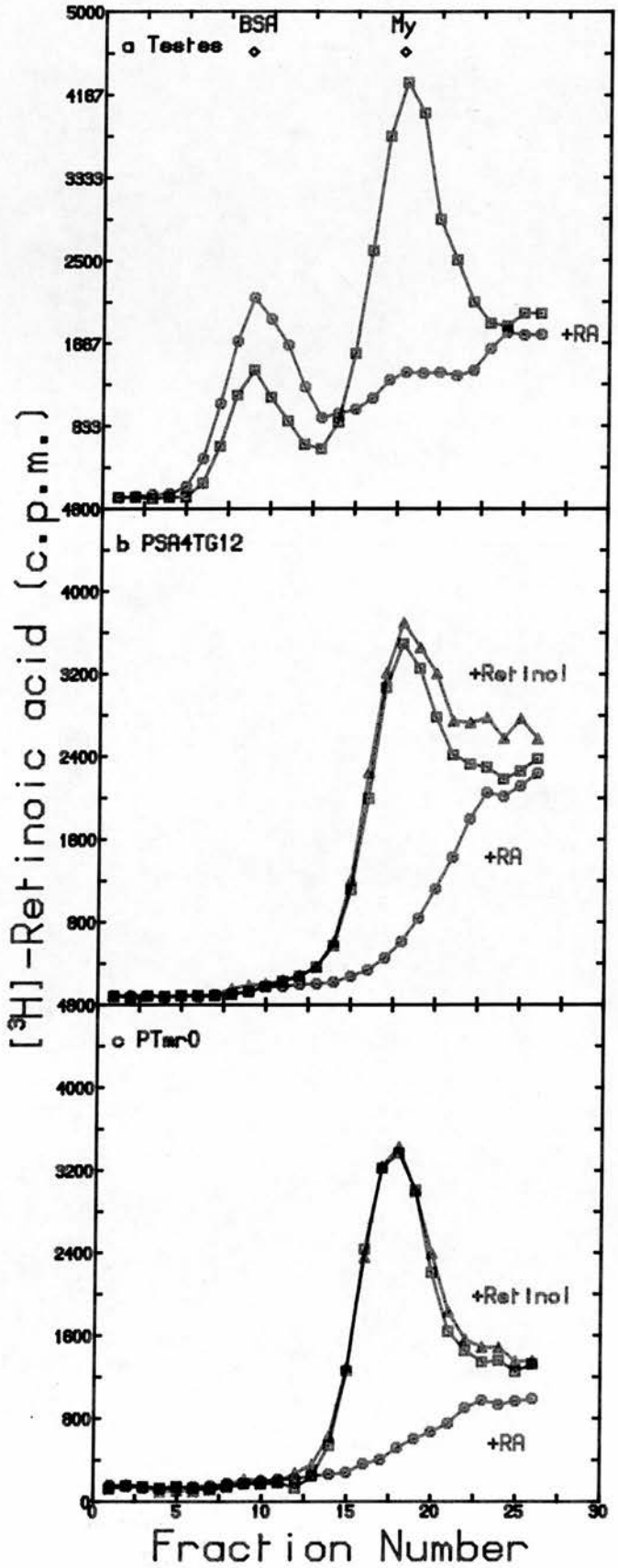
If CRABP does mediate the action of retinoids on gap juntions, variants producing little or no functional binding protein would be expected to show unimpaired metabolic cooperation in the presence of retinoic acid, i.e. the phenotype exhibited by PTmr0. It was therefore considered appropriate to determine whether PTmr0 expresed CRABP activity.

In tissue extracts retinoic acid binds specifically to CRABP and non-specifically to serum albumin (Section 1.5.). These two components can be resolved by density gradient centrifugation due to the difference in their sedimentation coefficients (Ong and Chytil, 1975). This is illustrated in Fig.3.3a which shows the binding profile on a sucrose gradient of [^{3}H]-retinoic acid in a crude cytoplasmic preparation of rat testes contaminated with serum proteins. Horse myoglobin (My), $\mathbf{S}_{20,w}$=2.04, and bovine serum albumin (BSA), $\mathbf{S}_{20,w}$=4.6, labelled with kenacid blue (Stanworth *et al*, 1961), were employed as markers in a separate gradient. The major peak of binding activity sediments in the 2**S** region of the gradient and is displaced by a 200-fold excess of unlabelled retinoic acid. This saturable component is CRABP (Ong and Chytil, 1975) and is well resoved from the minor, non-displaceable, contribution in the 4.6**S** region attributable to serum albumin.

Binding of [^{3}H]-retinoic acid to cytosols prepared from PSA4TG12 and PTmr0 was analysed by this procedure (Section 2.12.). A typical result for PSA4TG12 is shown in Fig.3.3b and for PTmr0 in Fig.3.3c. Both cytosols display a single symmetrical peak of activity with a sedimentation coefficient of approximately 2**S**. This peak is abolished in the presence of excess unlabelled retinoic acid. Binding of [^{3}H]-retinoic acid is unaffected by excess retinol, however. Retinal likewise fails to compete for the binding site (data not shown, but see Table

Fig.3.3 Sucrose Gradient Analyses of [^{3}H]-Retinoic Acid Binding

Sucrose gradient assays were performed as described in Section 2.12 on cytosols prepared from rat testes (a), PSA4TG12 (b) and PTmr0 (c). Red lines are the binding profiles of [^{3}H]-retinoic acid alone. Blue lines indicate binding in the presence of a 200-fold excess of unlabelled retinoic acid (+RA). Green lines show [^{3}H]-retinoic acid bound in the presence of a 200-fold excess of retinol. The sedimentation positions of bovine serum albumin (BSA) and myoglobin (My) are indicated in (a).


a Testes
BSA
My
+RA
b PSA4TG12
+Retinol
+RA
c PTmr-0
+Retinol
+RA
[3H]-Retinoic acid (c.p.m.)
Fraction Number
5000
4167
3333
2500
1667
833
4800
4000
3200
2400
1600
800
4800
4000
3200
2400
1600
800
0
0
5
10
15
20
25
30

4.7). The receptor is thus saturable and specific and fulfills the criteria for authentic CRABP (Ong and Chytil, 1975). As shown in Table 3.4, it is present in cytosols prepared from cells grown on feeders or grown on gelatin in STO feeder-conditioned medium, which reduces the extent of spontaneous differentiation (Smith and Hooper, 1983). No activity was detected in cytosols prepared from STO feeder layers alone.

Table 3.4
Estimations of CRABP Levels in EC Cell Cytosols by Sucrose Gradient Analyses of [^{3}H]-Retinoic Acid Binding

Cell Line	CRABP Activity (pmol [^{3}H]-retinoic acid bound/mg cytosol protein)
PSA4TG12 (f)	1.41,1.32
PTmr0 (f)	1.72,1.12
PSA4TG12 (g)	0.60
PTmr0 (g)	0.65
STO feeders	<0.1,<0.1
Nulli-1 (f)	0.59
Nulli-1 (g)	0.52
PC13.5 (g)	0.36,0.32

(f) Cultured on STO feeder layers
(g) Cultured on gelatinized substrata
Each figure is the result of a separate determination.

The levels of CRABP are similar for PSA4TG12 and PTmr0 and, for cultures on feeders, lie in the upper end of range of values reported by Matthaei *et al* (1983) for a variety of EC cell lines. The cells these workers analysed were all cultured on gelatin. On the basis of a single result for each cell line, CRABP activity in PSA4TG12 and PTmr0 appears lower when grown on gelatin rather than feeder layers. If this difference is real it probably reflects the less favourable culture environment and/or the occurrence of some differentiation in feeder-conditioned medium. Matthaei *et al* (1983) have shown that differentiated derivatives of teratocarcinoma often express lower specific activities of CRABP than embryonal carcinoma. Nulli-1 cells have a relatively high plating efficiency in the absence of feeder cells and undergo a very low level of spontaneous differentiation. CRABP activity in these cells appears similar whether cultured on feeders or on gelatin (Table 3.4).

3.3.2. Uptake of Retinoic Acid by PSA4TG12 and PTmr0

The resistance of PTmr0 to the effect of retinoic acid on metabolic cooperation could arise from a failure of these cells to take up retinoic acid from culture medium. This possibility was investigated using a radiolabelled tracer to study the association of retinoic acid with PTmr0 and PSA4TG12 cells.

The assays were performed in Leighton tubes so that the cultures could be maintained at 37°C by immersion in a water bath. Cells were grown on gelatinized surfaces in feeder-conditioned medium rather than on feeder layers, as trials indicated that feeder layers disintegrated during the cold washings required to terminate the incubations (Section 2.13.). Examination of EC monolayers on gelatin by light microscopy revealed that they remained intact throughout the assay. Moreover, the effect of retinoic acid on metabolic cooperation in PSA4TG12 and PTmr0 was analysed by uridine nucleotide transfer assays (Section 1.8.) in the absence of feeders (Smith *et al*, submitted). It is therefore appropriate to investigate uptake under such conditions and avoid possible complications such as the incorporation of retinoic acid by feeders and transfer to EC cells through gap junctions.

The time course of association of [^{3}H]-retinoic acid (8nM) with PTmr0 in CM containing 10% (v/v) newborn calf serum (NCS) or 0.15% (w/v) bovine serum albumin (BSA) is shown in Fig.3.4. There is a rapid uptake of radiolabel during the first first 10 min of incubation, which subsequently levels off. This pattern is in agreement with that described for other EC cells (Schindler *et al*, 1981; McCue *et al*, 1983) and is similar to that reported for melanoma cells (Lotan *et al*, 1977).

Association of [^{3}H]-retinoic acid with PTmr0 occurs more rapidly over a longer time period in medium supplemented with BSA rather than newborn calf serum. This may reflect degradation of the radiolabel, sequestration by binding to serum proteins, or competition for the uptake process by retinoids or other molecules present in serum. The difference is unlikely to be due to BSA, since the latter is present at a similar concentration in newborn calf serum (Handbook of Biological Data). As the measurable response was greater in the presence of 0.15% (w/v) BSA, this medium was employed for the comparison of uptake by PSA4TG12 and PTmr0.

Fig.3.4 Uptake of [^{3}H]-Retinoic Acid by PTmr0

Association of [^{3}H]-retinoic acid with PTmr0 was determined as described in Section 2.13. in CM containing 10% (v/v) newborn calf serum (NCS) or in serum-free medium supplemented with 1.5mg/ml bovine serum albumin (BSA).

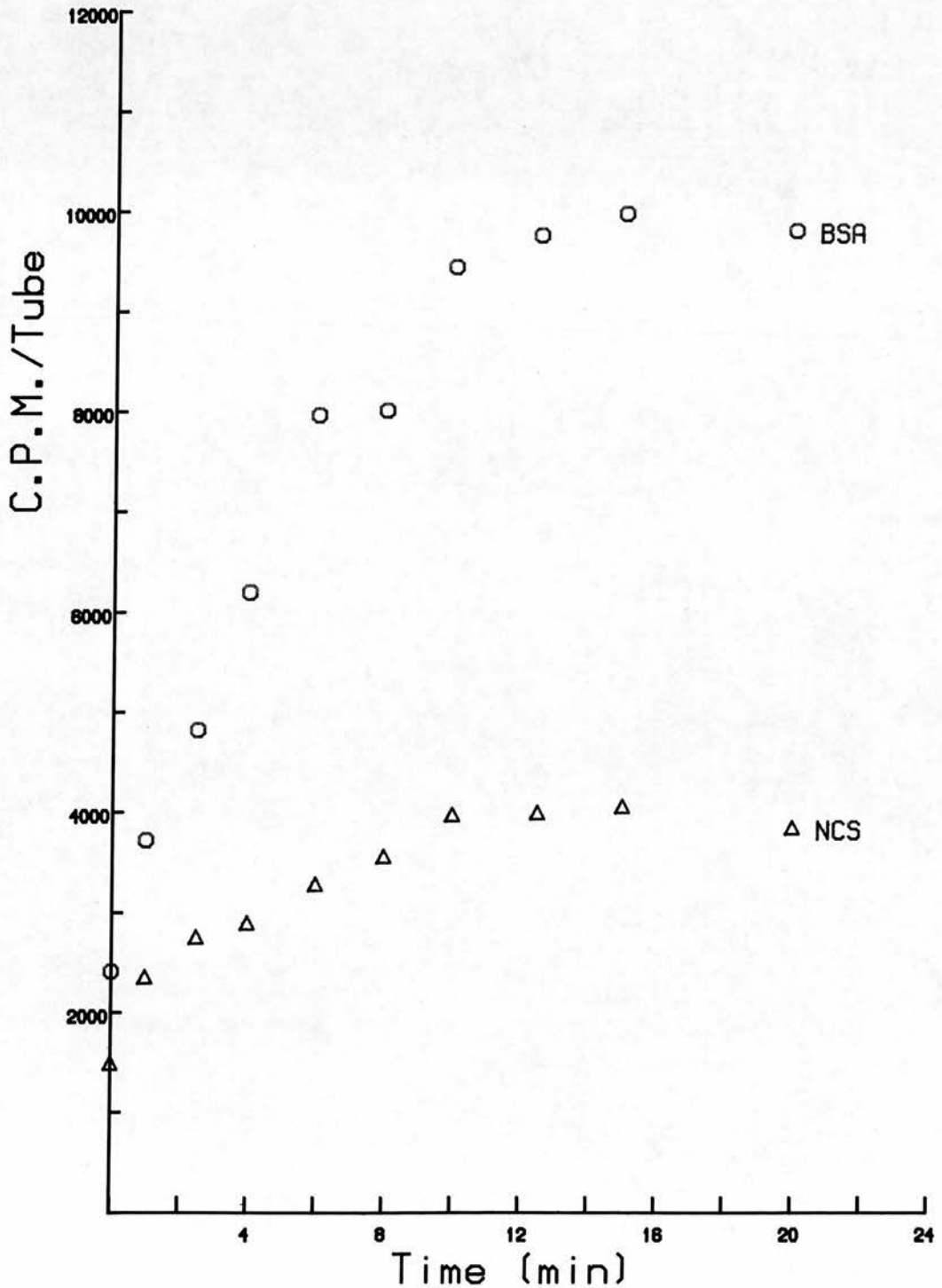
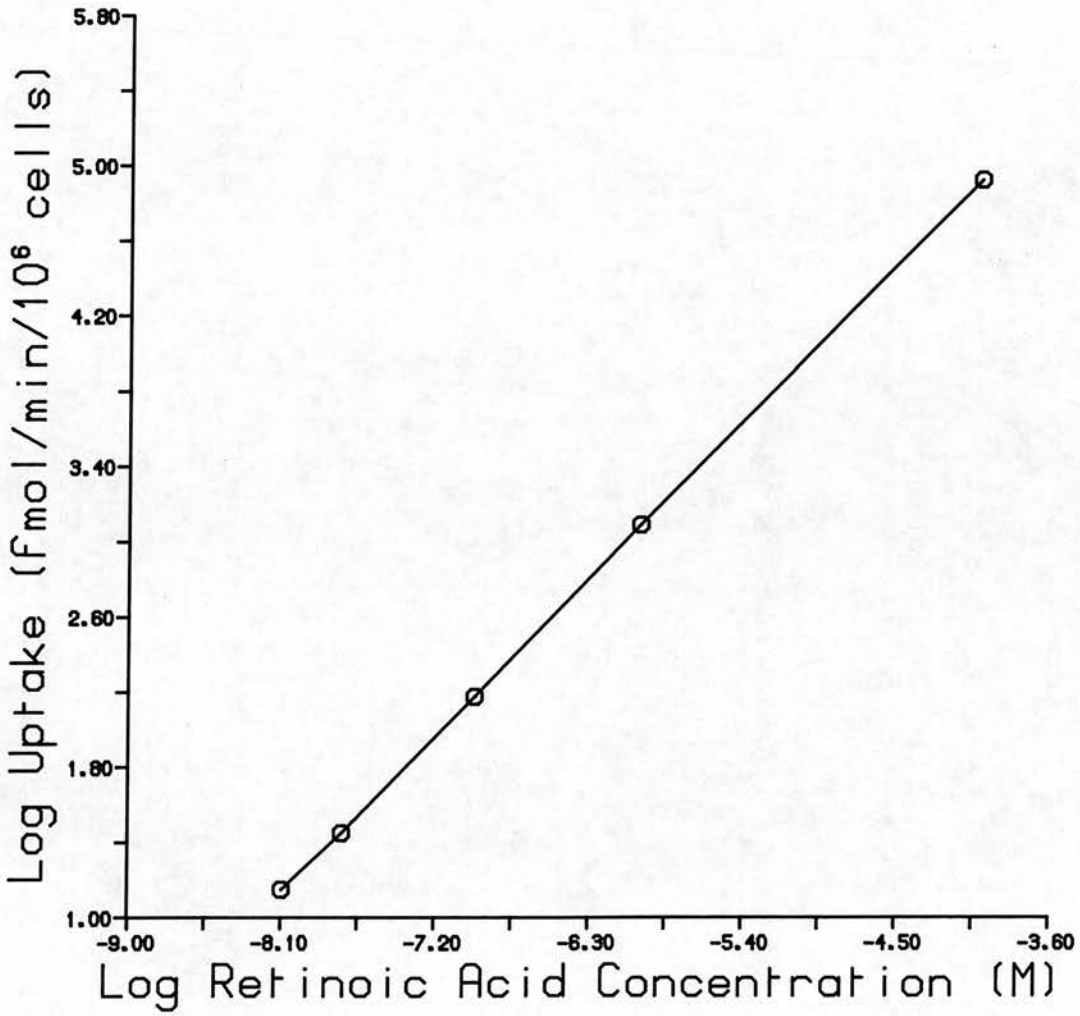

12000
10000
8000
6000
4000
2000
C.P.M./Tube
BSA
NCS
4
8
12
16
20
24
Time (min)

Fig.3.5 Log Plot of Initial Rates of Uptake of Retinoic Acid by PTmr0

Association of [^{3}H]-retinoic acid with PTmr0 was calculated as described in Section 2.13. For details of initial rate determination see text.

Fig.3.5 Log Plot of Initial Rates of Uptake of Retinoic Acid by PTmr0



Uptake of retinoic acid is approximately linear between 2-10 minutes (Fig.3.4). The inhibitory action of retinoic acid on metabolic cooperation is detectable within this time (Pitts *et al*, in press). Rates of association of [^{3}H]-retinoic acid with PSA4TG12 and PTmr0 in the presence of various concentrations of unlabelled retinoic acid were therefore calculated for this time period. Measurement of the association of [^{3}H]-retinoic acid with PSA4TG12 or PTmr0 was performed at 2 minute intervals (Section 2.13.) from 2-10 minutes inclusive after addition of retinoid. Rates of association were determined by linear regression analysis. The coefficient of determination, R^2, provides an estimate of the proportion of the variation in cell-associated c.p.m. due to variation in time (Sokal and Rohlf, 1981). The results are tabulated in Table 3.5.

Table 3.5
Initial Rates of Uptake of Retinoic Acid at Different Concentrations by PSA4TG12 and PTmr0

	PSA4TG12			PTmr0		
Conc. (nM)	$R^{2\sim}$	C*	F^	$R^{2\sim}$	C*	F^
8	.985	378.7	14.43	.878	374.5	14.27
18	.988	331.0	28.38	.961	327.2	28.05
108	.973	291.8	150.12	.894	290.9	149.66
10^3	.988	247.4	1187.95	.921	257.2	1235.01
10^5	.961	176.6	84121.40	.978	174.5	83121.09

~Coefficient of determination
*c.p.m./min/10^6 cells
^fmol/min/10^6 cells

For details see text.

PTmr0 and PSA4TG12 are similar in size (Smith *et al*, submitted), so clearly there is no detectable difference in the rate of association of retinoic acid with the two cell lines over the 10^{-8}-10^{-4}M concentration range. The rate of uptake increases linearly with concentration (Fig.3.5), as previously described for melanoma cells by Lotan *et al* (1977).

3.3.3. Cytoplasmic Binding of [^{3}H]-Retinoic Acid In Whole Cells

The above data (Section 3.3.2.) on association of retinoic acid with PTmr0 and PSA4TG12 refer to uptake by whole cells. This may comprise association of retinoic acid with the cell surface and non-specific membrane partitioning of the lipid-soluble retinoid (Fell *et al*, 1962; Lotan, 1980), in addition to

cytoplasmic internalization. It was therefore considered important to try and determine whether retinoic acid is taken into the cytoplasm of PTmr0 and forms complexes with CRABP *in vivo*.

This was investigated by preincubating log phase cultures of PSA4TG12 and PTmr0 grown on gelatin in feeder-conditioned medium (Smith and Hooper, 1983) with medium containing 40nM [^{3}H]-retinoic acid for 2 hours, then analysing the cytosols by sucrose gradient centrifugation as described in Section 2.12. The result of such an experiment on PTmr0 is shown in Fig.3.6. Similar results were obtained for PSA4TG12. The peak of radioactivity in the 2**S** region is abolished by incubation of the cytosols with $5x10^{-6}$M unlabelled retinoic acid before application to the gradient (data not shown).

Specifically bound [^{3}H]-retinoic acid represents appproximately 30% of the activity present in cytosols before extraction of unbound retinoid with charcoal/dextran. The concentration of [^{3}H]-retinoic acid in the untreated cytosols is around 18nM. This is less than apparent K_d values for the binding of all-*trans*-retinoic acid to CRABP in EC cell cytosols of 23nM and 38nM reported by Jetten and Jetten (1979) and Sherman *et al* (1983b) respectively. Therefore it is expected that less than 50% of the receptors will be occupied and most of the [^{3}H]-retinoic acid will be unbound, consistent with the observations.

However, only some 17% of the activity in unfractionated cell lysates was subsequently recovered in the cytosol (and about 1% in the nuclear fraction) in these experiments. As the cytosol is prepared in a single step (Section 2.12.) with little scope for loss, this implies that the bulk of retinoic acid in whole cells is either on the cell surface, membrane-associated, or in cell organelles. Indeed Sherman *et al* (1983a) have provided evidence for extensive association of retinoic acid with mitochondria. Therefore, it is conceivable that the observed cytoplasmic binding might not occur *in vivo*, but could merely be an artefact of cell disruption liberating previously sequestered retinoic acid and making it accessible to CRABP receptors. This possibility cannot be excluded from the available data. A different approach such as autoradiography would be required to demonstrate definitively that retinoic acid is present in EC cell cytoplasms.

Fig.3.6 Cytoplasmic Binding of [^{3}H]-Retinoic Acid by PTmr0

A cytosol fraction (Section 2.12.) was prepared from approximately 10^8 PTmr0 cells following incubation for 2 hours in medium containing 40nM [^{3}H]-retinoic acid. Unbound retinoid was extracted with charcoal/dextran and sucrose gradient analsysis performed as described in Section 2.12. Myoglobin was included in a separate gradient as a 2S marker.

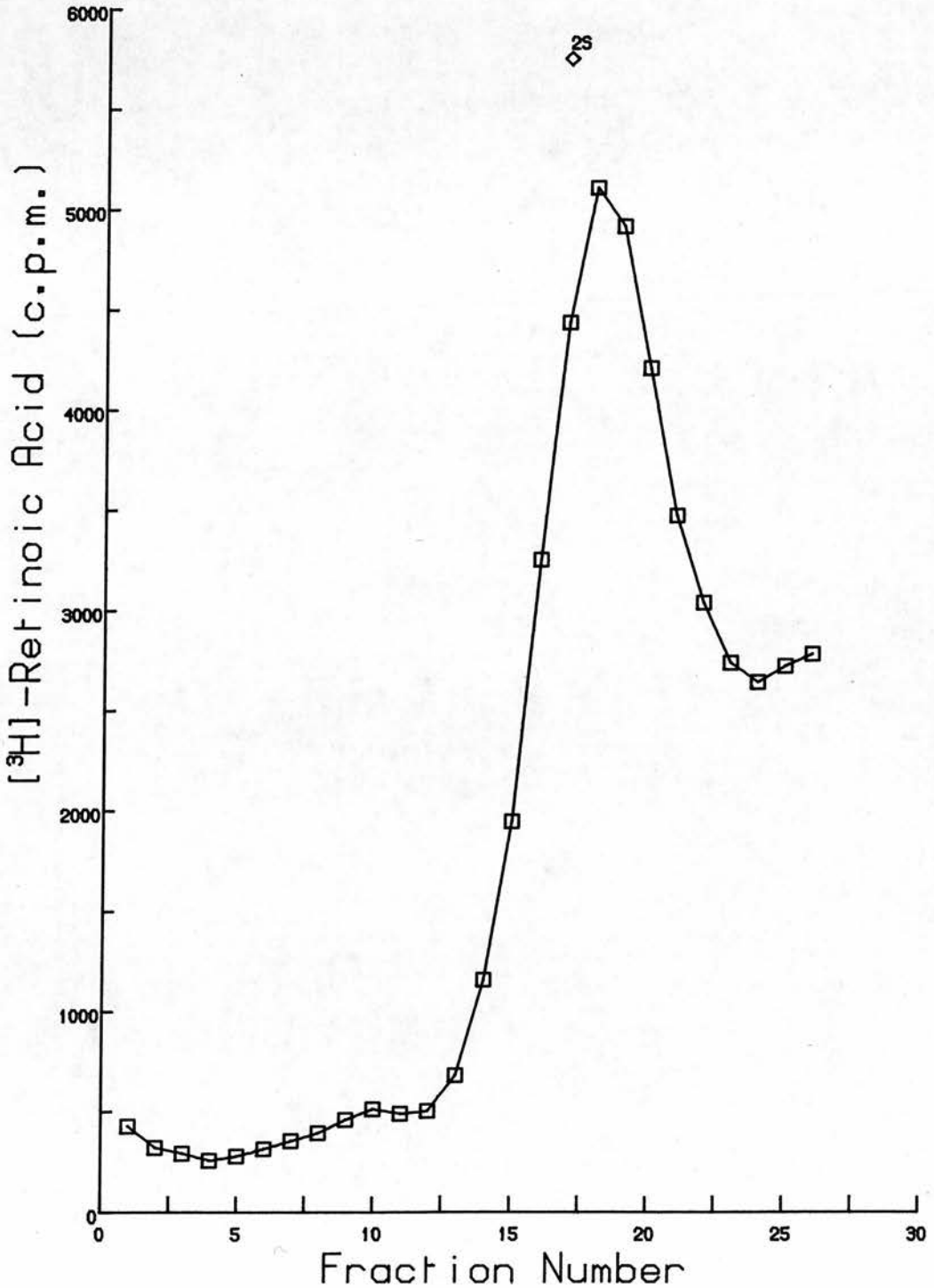
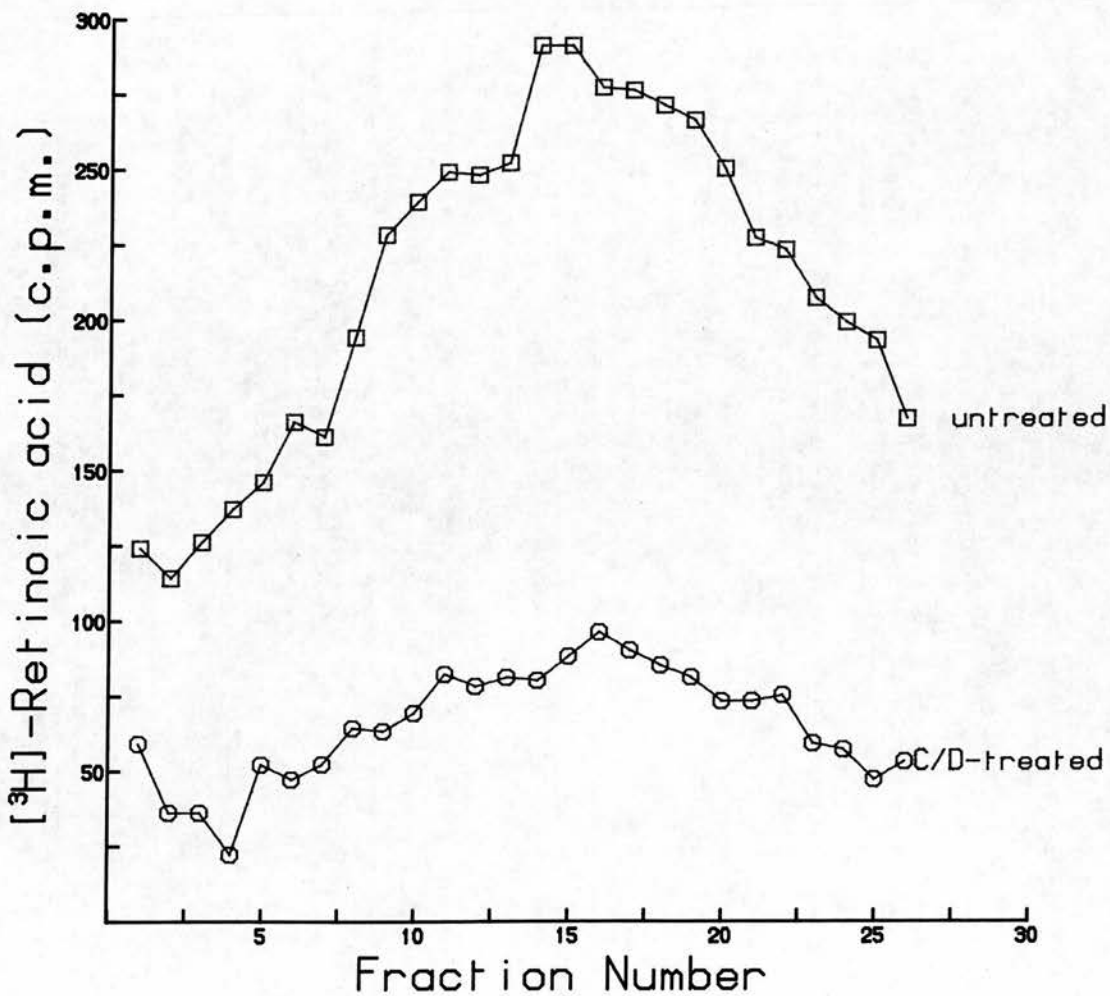

6000
5000
4000
3000
2000
1000
0
[3H]-Retinoic Acid (c.p.m.)
2S
0
5
10
15
20
25
30
Fraction Number

Fig.3.7 Nuclear Binding of [^{3}H]-Retinoic Acid by PTmr0

A nuclear fraction (Section 2.12.) was prepared from approximately 10^8 PTmr0 cells following incubation for 2 hours in medium containing 40nM [^{3}H]-retinoic acid. The fraction was divided into two samples. One (C/D-treated) was treated with charcoal/dextran (Section 2.12.), the other (untreated) was subjected to an equivalent dilution with PBS. Sucrose gradient analsysis was performed as described in Section 2.12.

Fig.3.7 Nuclear Binding of [^{3}H]-Retinoic Acid by PTmr0



3.3.4. Nuclear Binding of [^{3}H]-Retinoic Acid in Whole Cells

The steroid hormone model of retinoid action (discussed in Section 1.5.) predicts that the role of CRABP is to deliver retinoic acid to effector sites in the cell nucleus. It has been reported that [^{3}H]-retinoic acid bound to CRABP can be detected in nucleoplasm of the EC cell line PCC4aza1R following preincubation of whole cells with the radiolabelled ligand (Jetten and Jetten, 1979). Nuclear binding of retinoic acid by PSA4TG12 and PTmr0 were therefore compared.

These experiments were performed in conjunction with those on cytoplasmic binding in whole cells (previous Section). Nuclear fractions were subjected to sucrose gradient analysis both before and after treatment with charcoal/dextran. A typical result for PTmr0 is shown in Fig.3.7. Similar data were obtained for PSA4TG12.

Radioactivity is reduced by over 50% throughout the sucrose gradient by extraction with charcoal/dextran. This removes unbound ligand or ligand bound with low affinity (Korenman, 1968), but does not affect high affinity binding. No significant high affinity nuclear binding was detected in either PSA4TG12 or PTmr0 under the conditions obtaining in these experiments.

This result is in contrast to that of Jetten and Jetten (1979) but there are several possible explanations for this. Firstly, these workers used a different EC cell line, PCC4aza1R, which had been selected for the ability to grow in retinoic acid (Jetten *et al*,1979) and may have different properties from PSA4-derived EC cells. Secondly, they employed a much higher concentration of [^{3}H]-retinoic acid during the preincubation period (10^{-6}M compared with $4x10^{-8}$M) which should have ensured saturation of CRABP, unlike the experiments described here (see previous Section). Thirdly, no indication is given of cell equivalents, or protein or DNA concentrations; it is possible that a specific binding component might be resolved in PSA4TG12 and PTmr0 nuclear fractions if these were more concentrated. More fundamentally, they make no assessment of the purity of their nuclear fraction. It is possible that their finding of nuclear retinoic acid-CRABP complexes is an artefact arising from contamination of the nuclear fraction with cytoplasmic material, particularly in view of conflicting findings from other systems (see Section 1.5.2.).

Two other factors must be taken into consideration with regard to nuclear binding of retinoic acid. One is the length of preincubation. This could be important if accumulation of retinoic acid and/or retinoic acid-CRABP complexes by PSA4 nuclei is relatively slow. One hour incubation periods were employed in these experiments because this period has been used by other workers (Wiggert *et al*, 1977; Jetten and Jetten, 1979) and the effect of retinoic acid on gap junctions is rapid (Pitts *et al*, in press). However, less than 1% of total radioactivity was recovered in the nucleoplasm. This proportion may have been greatly increased on incubation for a longer period and/or with a higher concentration of radiolabel. Sherman *et al* (1983a), using similar fractionation procedures, have reported that after 16 hours incubation with 1.7×10^{-6}M [^{3}H]-retinoic acid, 45% of radioactivity associated with Nulli-1 cells is present in the nuclei (and only 10% in the cytosol).

The other consideration is the possibility of extraction of nuclear material into the aqueous phase, i.e. the cytosol, on homogenisation. This has led to controversy over the interpretation of some experiments on steroid hormone receptors, for example (Szego and Pietras, 1985).

It follows from these arguments that no conclusions can be drawn concerning the nuclear translocation of retinoic acid in PSA4TG12 or PTmr0 nuclei *in vivo*.

Binding of [^{3}H]-retinoic acid by isolated nuclei or nuclear fractions was not investigated as previous reports on EC cells (Jetten and Jetten, 1979), retinoblastoma (Wiggert *et al*, 1977) and adenocarcinoma (Mehta *et al*, 1982) indicate that no specific binding occurs in the absence of cytoplasmic extracts.

3.3.5. Electron Spin Resonance Studies On Plasma Membranes

Retinoids are lipid-soluble and consequently can partition into cell membranes (Fell *et al*, 1962; Lotan, 1980). As they are amphipathic molecules, this can result in membrane labilization, lysozomal enzyme release and cell lysis (Roels *et al*, 1969; Rundell *et al*, 1974; Wang *et al*, 1975). High concentrations of retinoid have been shown to decrease the 'apparent' microviscosity of membranes of erythrocytes (Meeks *et al*, 1981) and differentiated teratocarcinoma cells (Jetten *et al*, 1982). Gap junctions are integral parts of the plasma membrane, so it is conceivable that perturbation of membrane structure could underlie the disruption of junctional communication caused by

retinoic acid. Such an explanation would account for the high levels of retinoic acid required for this effect, levels which are both toxic (Section 3.2.2.) and several orders of magnitude higher than the physiological concentrations at which other responses are induced, including differentiation of embryonal carcinoma. The concept of membrane fluidity, which refers to the dynamics of fatty acyl chains of membrane phospholipids, has proved very useful in the analysis of the physical properties of biomembranes (Stubbs, 1983). It is influenced by chain length and degree of *cis*-unsaturation, and by interaction with other membrane components. Electron spin resonance spectroscopy (ESR) was used to examine the fluidity of cell membranes of PSA4TG12 and PTmr0 and the effects of retinoic acid thereon.

Plasma membrane fractions were prepared from log phase cultures by differential centrifugation of cell lysates (Thom *et al*, 1977) as described in Section 2.14. Examination of the preparations by electron microscopy revealed that they were composed of membrane vesicles with no contaminating subcellular organelles (Plate 3.3 inset). As EC cells contain very little endoplasmic reticulum and only rudimentary Golgi (Martin, 1975; Speers *et al*, 1979; Lo and Gilula, 1980b), it was concluded that these vesicles consisted primarily of plasmalemma.

ESR measurements were performed on pooled batches of membrane from at least two separate preparations. Plate 3.3 shows the spectrum obtained from PTmr0 membranes at 25°C. A minor component of the spectrum corresponds to free spin label in aqueous solution, but most of the signal is from probe incorporated into the membrane. Information on membrane fluidity is provided by the order parameter, **S**, which is a measure of the angular range of rotational motion of the nitroxide moiety around the long axis of the fatty acyl chain (Knowles *et al*, 1976). The approximate order parameter, $\mathbf{S}_{app}$, is calculated from the line splittings as described in the Appendix.

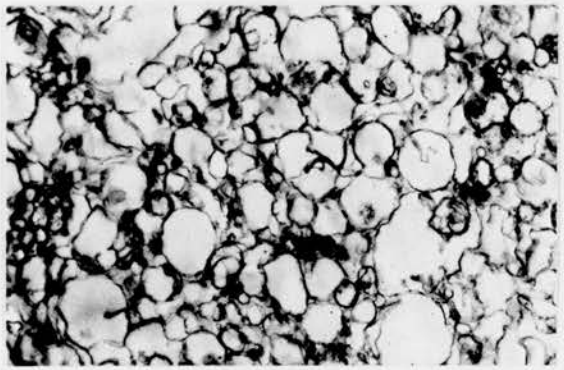
Table 3.6 details values of $\mathbf{S}_{app}$ for 5-doxyl stearate incorporated into plasma membranes of PSA4TG12 and PTmr0 in the absence of retinoic acid in three separate experiments.

Plate 3.3 ESR Spectrum of 5-Doxyl Stearate in PTmr0 Membrane Suspension

The spectrum was obtained as described in Section 2.15. S_{app} was calculated from the separations in Gauss (G) of the maximum (1,4) and minimum (2,3) extrema of the spectra as detailed in the Appendix. The minor resonances attributable to free nitroxide spin label in aqueous solution are indicated by x and y.

Inset. Electron Micrograph of PSA4TG12 Membranes

For details of preparation see Section 2.14.
Magnification: 8000



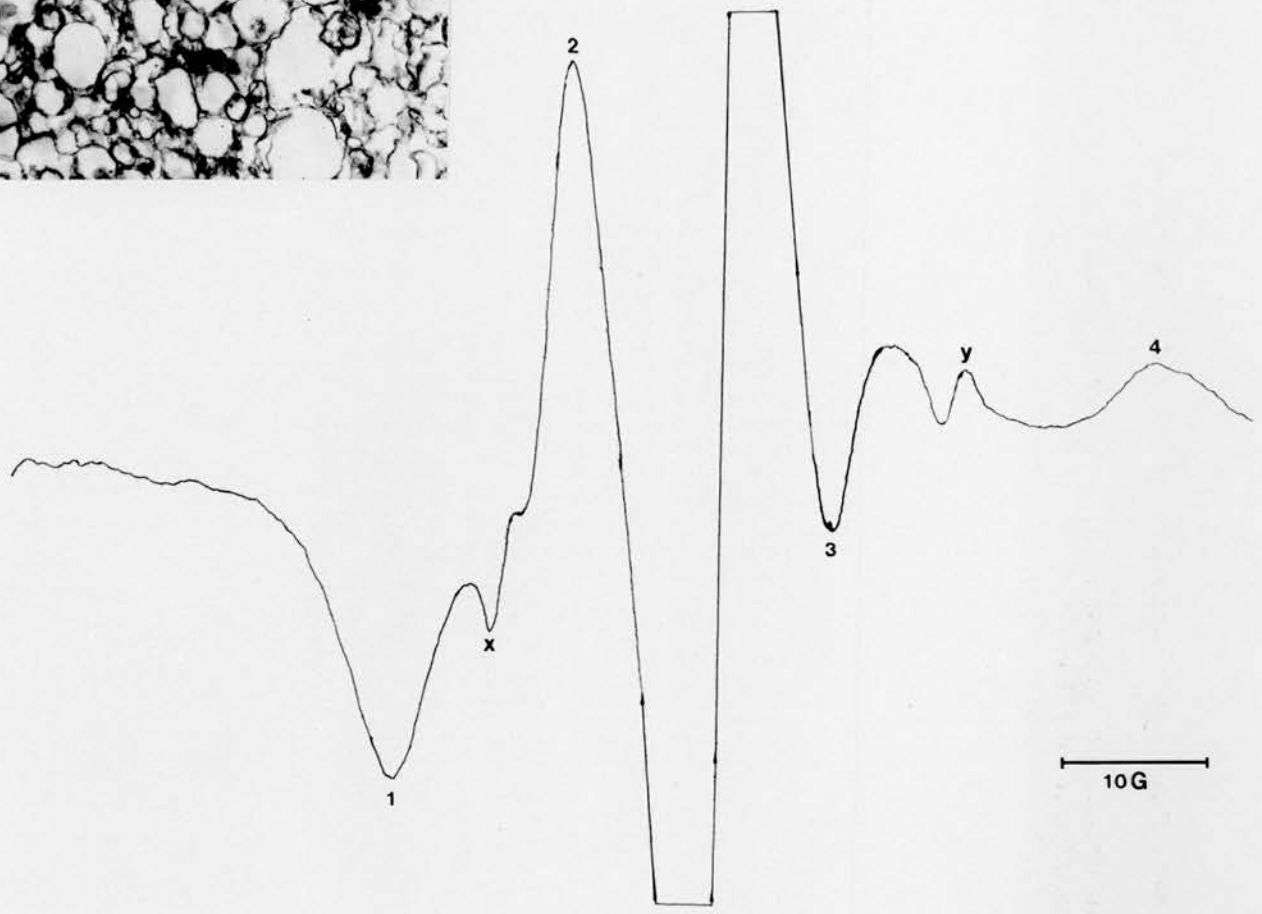

2
y
4
3
x
1
10 G

Table 3.6
Approximate Order Parameters (S_{app}) for 5-Doxyl Stearate in PSA4TG12 and PTmr0 Plasma Membranes at 25°C

	PSA4TG12	PTmr0
Expt.1*	0.618	0.617
Expt.2	0.644	0.626
Expt.3	0.646	0.654
Mean±s.e.m.	0.636±0.009	0.632±0.011
Mean±s.e.m. for all samples	0.634±0.010	

Clearly no significant difference in the properties of the two cell membranes at 25°C is detected by ESR. The value of S_{app} is relatively low, indicating that these cells have quite fluid membranes, as previously reported in fluorescence studies on EC cell membranes (Jetten *et al*, 1982; Searls and Edidin, 1981).

The effect of 10^{-4}M retinoic acid on the membranes was also investigated (Table 3.7).

Table 3.7
Effect of 10^{-4}M Retinoic Acid on S_{app} for 5-Doxyl Stearate in PSA4TG12 and PTmr0 Membranes at 20°C

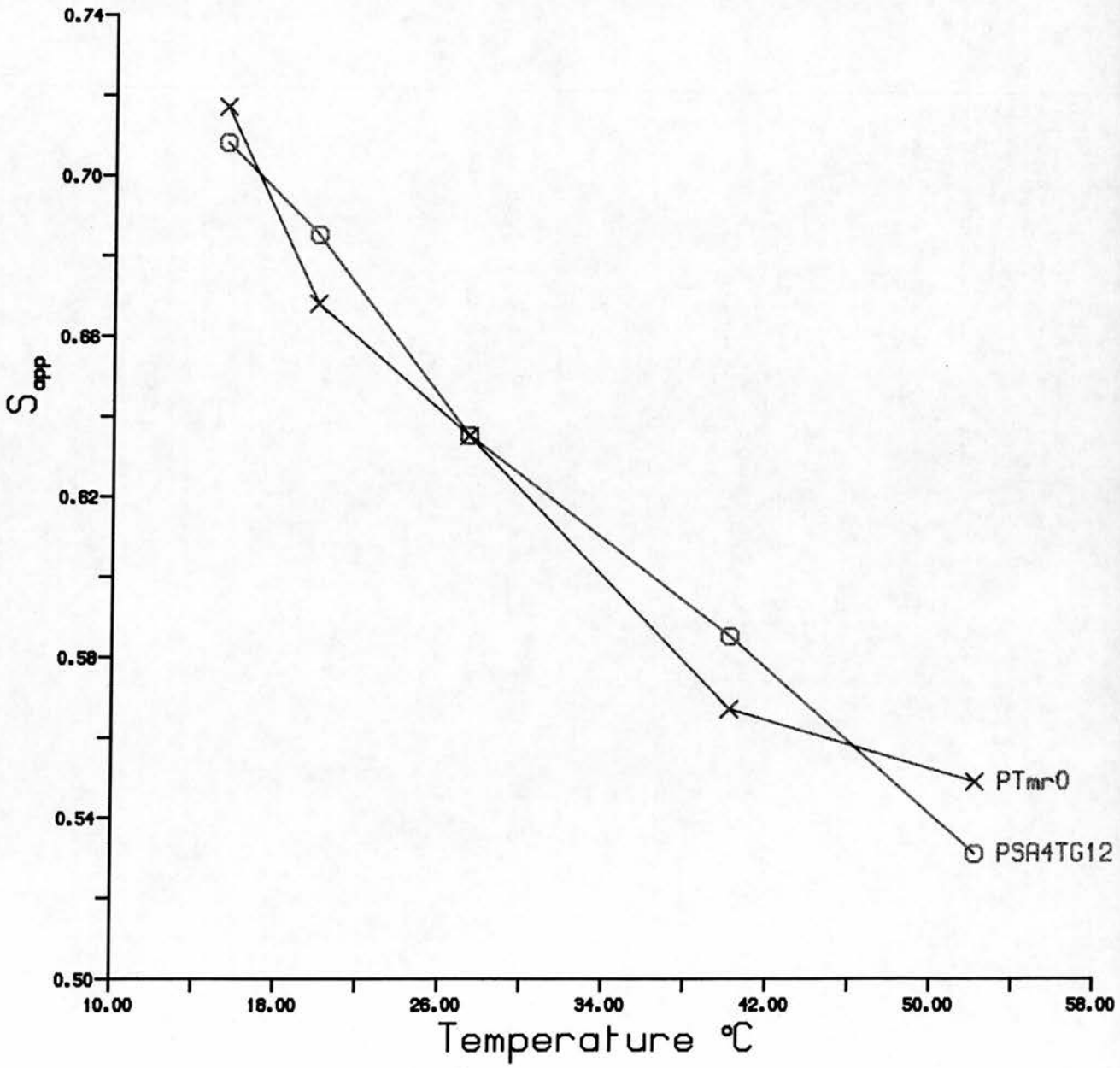
	PSA4TG12	PTmr0
Control	0.713	0.685
Retinoic acid	0.685	0.668

In both cell lines 10^{-4}M retinoic acid induces small increases in membrane fluidity as measured by reductions in the order parameter. The response is so small that any difference between the two membrane samples at this temperature would be difficult to detect. The fluid properties of biological membranes are highly temperature-dependent, however. S_{app} values were therefore determined for the two cell membranes in the presence of retinoic acid at a series of temperatures spanning the physiological temperature. The results are diagrammed in Fig.3.8.

S_{app} decreases, i.e. membrane fluidity increases, with increasing temperature in a similar manner for both cell membranes. Thus the physical interaction of retinoic acid with plasma membranes, as measured by this highly sensitive parameter, does not differ significantly between the two cell lines.

Fig.3.8 Temperature-Dependence of S_{app} in the Presence of 10^{-4}M Retinoic Acid

The approximate order parameter, S_{app}, was determined as described in Section 2.15 (see also Appendix) for the 5-doxyl stearate spin label incorporated into plasma membranes prepared (Section 2.14.) from PSA4TG12 (blue line) or PTmr0 (red Iine) in the presence of 10^{-4}M retinoic acid at different temperatures.


0.74
0.70
0.66
0.62
0.58
0.54
0.50
S_{app}
10.00
18.00
26.00
34.00
42.00
50.00
58.00
Temperature °C
PTmr0
PSA4TG12

3.4. DISCUSSION

Retinoic acid has pronounced effects on junctional communication between all cells so far examined, including embryonal carcinoma (Pitts *et al*, 1981; Smith, 1984; Shuin *et al*, 1983; Walder and Lutzelschwab, 1984; Pitts *et al*, in press). Amongst the various roles suggested for gap junctions is the proposal that they have an important function in embryonic development as channels through which developmental signals pass (Furshpan and Potter, 1968). Inhibition of intercellular communication has been invoked as a possible explanation for the profound effects of retinoic acid on developmental systems (Maden, 1982; Pitts *et al*, in press; see Section 1.8.). However, most reports on the action of retinoic acid on metabolic cooperation find that inhibition is only manifest at very high concentrations ($>10^{-5}$M in serum-containing medium). These levels far exceed those obtaining *in vivo*, rendering this unlikely as a physiological effector mechanism. Moreover, most other responses to retinoic acid, notably differentiation of EC cells, are induced at far lower, more physiological, concentrations.

Recent work by Walder and Lutzelschwab (1984) suggests one way in which this discrepancy might be resolved. These workers, using a different experimental protocol, found that exposure to retinoic acid for longer periods than previously employed enabled detection of inhibitory effects on junctional permeability at concentrations as low as 10^{-6}M. Lower concentrations were not investigated, nor incubation periods longer than 24 hours, so it remains possible that retinoic acid could exert some inhibitory action on metabolic cooperation at similar concentrations to the minimum required for induction of differentiation (see Chapter 4 for further discussion).

Three hypotheses thus present themselves:-

1. The multiple effects of retinoic acid are a consequence of the inhibition of junctional communication.
2. The actions of retinoic acid are not mediated via inhibition of junctional communication, but share a common mechanism in which the effect on metabolic cooperation has a higher threshold than the other responses.

3. Retinoic acid has more than one mode of action. Specifically, its effects on junctional communication are independent of its effects on cell differentiation.

PTmr0, with its unique resistance to the action of retinoic acid on junctional communication, provides a novel means of discriminating between these three alternatives and possibly shedding some light on the underlying biochemistry.

The results reported in Section 3.2.1. demonstrate unequivocally that retinoic acid induces differentiation of PTmr0 at concentrations as low as 10^{-8}M. This appears to preclude the involvement of inhibited junctional communication in the inductive mechanism for EC cell differentiation and thus to eliminate hypothesis 1. One qualification must be made to this conclusion, however. This is that although retinoic acid has no detectable effects on on metabolic cooperation of PTmr0 over incubation periods up to 6 hours (Smith, 1984), maximal induction of differentiation requires exposure to the retinoid for 24 hours (Rayner and Graham, 1982). It is conceivable that some effect on junctional communication could become manifest over this longer period. This possibility has not been directly tested. Given the complete resistance displayed over 4 hour incubations in 10^{-4}M retinoic acid, however, it seems highly unlikely that any effect manifested after a longer exposure would extend to concentrations as low as 10^{-8}. Moreover, evidence will be presented in Chapter 4 that prolonged exposure does not render EC cells more sensitive to the effects of retinoic acid on metabolic cooperation (Section 4.2.). In addition, although the maximal response is only induced after 24 hours, many EC cells become committed to differentiate after briefer exposure to retinoic acid (Rayner and Graham, 1982; Mummery *et al*, 1984).

In theory, the data on induction of plasminogen activator (Section 3.2.1.) could be explained by a change in the pattern of differentiation as well as by a reduction in the amount of differentiation, since not all differentiated cells produce plasminogen activator. However, retinoic acid-induced differentiation of PSA4 and its derivatives in BRL-medium is restricted almost wholly to parietal endoderm-like cells which do release plasminogen activator (Section 5.3.6.) and the fibrin overlay results are in very good agreement with morphological assessments of the proportion of differentiated colonies for and PTmr0. Although the possibility of some differentiation of PTmr0 into non-plasminogen activator-producing cells cannot be formally excluded by this methodolgy, it is

more likely that the net amount of differentiation is lower for PTmr0 than PSA4TG12. In either event, there is a small but significant difference between the responses of the two cell lines. This means that no definite conclusion can be reached regarding hypotheses 2 and 3.

The altered sensitivity of the differentiation response could be causally related to the altered sensitivity of junctional communication (hypothesis 2). A 10-fold decrease in the sensitivity of the latter would result in little or no effect being detected at 10^{-4}M retinoic acid, as PSA4TG12 shows normal cooperation at 10^{-5}M retinoid (Smith *et al*, submitted). Higher concentrations than 10^{-4}M have not been investigated as this is the limit of solubility in serum-containing medium.

Alternatively, the two changes in phenotype could be coincidental (hypothesis 3). The protocol employed to isolate PTmr0 (Smith *et al*, submitted) entailed a prolonged period of culture in 10^{-4}M retinoic acid. Even in the presence of an STO feeder layer, there is considerable cell death and a high proportion of the survivors differentiate in the presence of this level of retinoic acid (Smith *et al*, submitted). This protocol therefore imposes selective pressure for EC cells with decreased sensitivity towards the differentiation-inducing properties of retinoic acid in addition to that for cells capable of metabolic cooperation in 10^{-4}M retinoic acid.

These two explanations for the phenotype of PTmr0 could only be discriminated by isolation of a revertant with normal cooperation properties. This might be done via a thioguanine "kiss of death" selection (see Section 1.8.) in the presence of 10^{-4}M retinoic acid (though such a protocol would also select for mec^- variants). Hypothesis 2 predicts that such a revertant, if obtained, would display a wild-type dose-response curve for induction of differentiation, whereas hypothesis 3 predicts that it would retain the altered responsiveness of PTmr0.

The data on retinoic acid toxicity (Section 3.2.1.) render it unlikely that the differences in metabolic cooperation observed between PTmr0 and PSA4TG12 arise from increased viability of the former. It is apparent that prolonged exposure to concentrations of retinoic acid which inhibit junctional communication in PSA4TG12 is acutely toxic to both cell lines (Fig.3.2). Toxic effects under the conditions obtaining in the uridine nucleotide transfer assays

of metabolic cooperation (Smith *et al*, submitted), namely 3-6 hour incubations, high cell density and the presence of residual feeder cells, have not been directly investigated, however.

As with the results on differentiation, it seems reasonable to conclude that inhibition of junctional communication is not the means by which the toxic actions of retinoic acid are effected. Interpretation of the apparent decrease in susceptibility to retinoic acid shown by PTmr0 is likewise similar to that for its decreased sensitivity to the induction of differentiation: it could be related to, or coincidental with, the variant metabolic cooperation phenotype. It is striking, however, that both differentiation and cell death dose response curves are shifted towards higher concentrations of retinoic acid. It is also noteworthy that toxicity in BRL-medium only becomes significant at concentrations of retinoid inducing differentiation of the entire population i.e $>10^{-8}$M for PSA4TG12 and $>10^{-7}$M for PTmr0. This provides circumstantial support for the suggestion that these two phenomena may be interconnected (see below Sections 4.5. and 4.6.).

Retinoic acid inhibits intercellular communication through preformed gap junctions (Walder and Lutzelschwab, 1984; Pitts *et al*, in press). The action is rapid, occurring within 5 minutes (Pitts *et al*, in press), and is fully and rapidly reversible (Pitts *et al*, 1981; Pitts *et al*, in press). These observations suggest that retinoic acid acts by closing junctional channels rather than preventing formation or causing breakdown of gap junctions. Electron microscopic examination reveals that gap junctions isolated from retinoic acid-treated cells are indistinguishable from those of control cultures (Pitts *et al*, in press) and similar amounts of junctional protein are recovered from treated and control populations (Hamilton and Pitts, 1981).

The biochemical studies on PTmr0 presented in Section 3.3 have focussed on three areas of interaction:-

1. Receptor binding
2. Cellular uptake
3. Plasma membrane labilization

The data on [^{3}H]-retinoic acid binding to cytosols (Section 3.3.1.) demonstrate

that PTmr0 possesses high levels of CRABP activity, whilst Sections 3.3.2. and 3.3.4. indicate that it is probable that exogenous retinoic acid is internalized by PTmr0 and that retinoic acid-CRABP complexes are formed *in vivo*. An involvement of CRABP in the effect of retinoic acid on junctional transfer is not precluded by these experiments, however, as it is possible either that PTmr0 CRABP is defective such that retinoic acid-CRABP complexes are not functional, or that there is a lesion in some post-receptor part of the mechanism. A role for CRABP in retinoid-mediated inhibition of junctional communication can only be formally excluded by demonstration of the inhibitory effect in cells which lack CRABP.

The variant phenotype of PTmr0 does not appear to arise from any gross alteration in plasma membrane properties (Section 3.3.5). It remains possible, however, that there are localized changes in lipid domains associated with gap junctions [or perhaps with ion pumps, as it is known that changes in intracellular Ca^{2+} or pH affect junctional permeability (Hooper and Subak-Sharpe, 1981; Hooper, 1982b)] which render them resistant to perturbation by retinoic acid. Alternatively, the junctional protein itself could be modified in PTmr0.

One theory of retinoid action which has not been investigated in this study is the proposal that their primary action is on glycosylation of membrane components, leading to altered cell surface properties (see Section 1.5.). De Luca (1977) has shown that retinol can participate in glycosyl transfer reactions in biological membranes, but no such role has been demonstrated for retinoic acid, and it is not converted to retinol *in vivo*. Retinoic acid does enhance glycosylation of specific cell surface glycoproteins in murine melanoma cells (Lotan *et al*, 1983) but these changes occur over a period of days and result from increased enzyme activity rather than any direct involvement of retinoic acid in sugar transfer (Lotan *et al*, 1984). Such long-term effects could not mediate the rapid, reversible response of junctional communication to retinoic acid.

CHAPTER 4

FURTHER STUDIES INTO THE EFFECTS OF RETINOIC ACID ON MURINE EMBRYONAL CARCINOMA CELLS

4.1. INTRODUCTION

Retinoic acid has pleiotypic effects on EC cells as discussed in Chapter 1. In Chapter 3 the relationships between three of these actions, namely induction of differentiation, induction of cell death and inhibition of metabolic cooperation, were investigated in the variant cell line PTmr0. In this Chapter the study is extended to other embryonal carcinoma cell lines.

One mechanism by which retinoic acid might trigger the differentiation of embryonal carcinoma cells is by inhibiting junctional communication between neighbours and thus "isolating" individual cells. The structural requirements for induction of EC cell differentiation (Jetten and Jetten, 1979; Sherman *et al*, 1983b; Strickland *et al*, 1983) and inhibition of metabolic cooperation (Pitts *et al*, in press) by retinoids appear similar (see also Section 3.3.1.). However, the differentiation response of EC cells is detectable at concentrations of retinoic acid as low as 10^{-9}M (Strickland and Mahdavi, 1978; Jetten *et al*, 1979) whereas inhibition of metabolic cooperation has usually been found only at concentrations greater than 10^{-5}M except under serum-free conditions (Smith, 1984; Pitts *et al*, in press). As discussed in Section 3.4., Walder and Lutzelschwab (1984) have provided evidence that lower concentrations of retinoic acid may be effective inhibitors of junctional communication if cells are exposed to the retinoid for a longer period. Maximal induction of EC cell differentiation requires at least a 24 hour exposure to retinoic acid (Rayner and Graham, 1982). Section 4.2. is a study into the effect on metabolic cooperation of prolonged exposure of PC13.5 embryonal carcinoma cells to retinoic acid.

All non-variant feeder-independent EC cells appear to undergo differentiation on exposure to retinoic acid, although in some cases there are high levels of cell mortality (Strickland and Mahdavi, 1978; Jetten *et al*, 1979). At the onset of this investigation only two variants had been isolated which did not differentiate in response to retinoic acid (Schindler *et al*, 1981), though others have since been reported (Jones-Villeneuve *et al*, 1983; McCue *et al*, 1983;

Wang and Gudas, 1984; Moore *et al*, 1985). EC cells refractory to the differentiation-inducing action of retinoic acid could be employed to examine the relationship between this and other responses in a similar manner to the study on PTmr0 described in Chapter 3. Variant EC cells with deficient differentiation responses might also be used to investigate the mechanisms of commitment and differentiation and evaluate the relevance of the retinoid pathway to events occurring in embryonic development. Furthermore, the variants obtained by Schindler *et al* lack, or have greatly reduced, CRABP activity (Schindler *et al*, 1981; Sherman *et al*, 1981). Such cells are potentially of considerable use in elucidating the role of CRABP, not only in differentiation *per se* (McCue *et al*, 1983, 1984a,b), but in retinoid activity generally. Thus a variant lacking CRABP could be used to determine conclusively whether the binding protein is involved in inhibition of metabolic cooperation (see Section 3.4.). Section 4.3. describes the isolation and characterisation of variant EC cells with a greatly reduced differentiation response to retinoic acid. The results of assays for CRABP in a series of cytosols prepared from a variety of EC cells are presented in Section 4.4.

In section 4.5. there is an investigation into the effects of retinoic acid on PT2md1, a variant EC line with reduced metabolic cooperation and differentiation capacity (Smith, 1984).

4.2. THE EFFECT OF PROLONGED EXPOSURE TO RETINOIC ACID ON JUNCTIONAL COMMUNICATION OF PC13.5 EMBRYONAL CARCINOMA CELLS

Previous studies into the effects of retinoic acid on the metabolic cooperation of EC cells (Smith, 1984; Smith *et al*, submitted) have utilised the uridine nucleotide transfer assay of Pitts and Simms (1977) (see Section 1.8.). This measures both the rate of formation of gap junctions and the permeability of the junctions once formed over a relatively short incubation period (routinely 4 hours). The sensitivity of the assay declines on longer incubation as the specific activity of the nucleotide pool decreases with time. Retinoic acid is normally added to the recipient population immediately prior to addition of the donors. It would be feasible to preincubate the recipients and/or the donors with retinoic acid for any desired period. With increasing time, however, more cells will become committed to differentiate, reaching a maximum after 24 hours (Rayner and Graham, 1982) by which time some characteristics of

differentiated cells, such as expression of EGF receptors (Rees *et al*, 1979), will be manifest. Differentiated teratocarcinoma cells may display altered junctional communication properties from EC cells (Lo and Gilula, 1980c; M.L. Hooper, personal communication). Thus any difference in metabolic cooperation between treated and untreated populations could be a consequence of some differentiation event rather than a direct response to retinoic acid *per se*. It might be possible to circumvent such a problem by using variant EC lines which do not differentiate in retinoic acid. If junctional communication between such cells responded to retinoic acid in the same way as cooperation between differentiating cells, it would confirm that the effect was manifested in the EC cells. (In the event of a different response, however, it would not be clear whether this arose from a failure to differentiate or was an altered property of the variants themselves.) The isolation of differentiation-defective lines and a preliminary investigation of their cooperation properties is described in the following Section.

These lines were not available at the outset of this work, however, so the modified ouabain rescue protocol described in Section 2.11. was employed to estimate metabolic cooperation in the presence of retinoic acid. This assay has the practical advantage in the present case that the STO feeder layers partially inhibit the induction of differentiation by retinoic acid (Ogiso *et al*, 1982; Rosenstraus *et al*, 1984). This effect is at least partly mediated via the medium and is not a result of inactivation of the retinoid (Smith and Hooper, 1983). The results of two ouabain rescue experiments on the EC line PC13.5 are presented in Table 4.1 and summarized diagrammatically in Fig.4.1.

Table 4.1
Ouabain Rescue Assays on PC13.5 in the Presence of Retinoic Acid

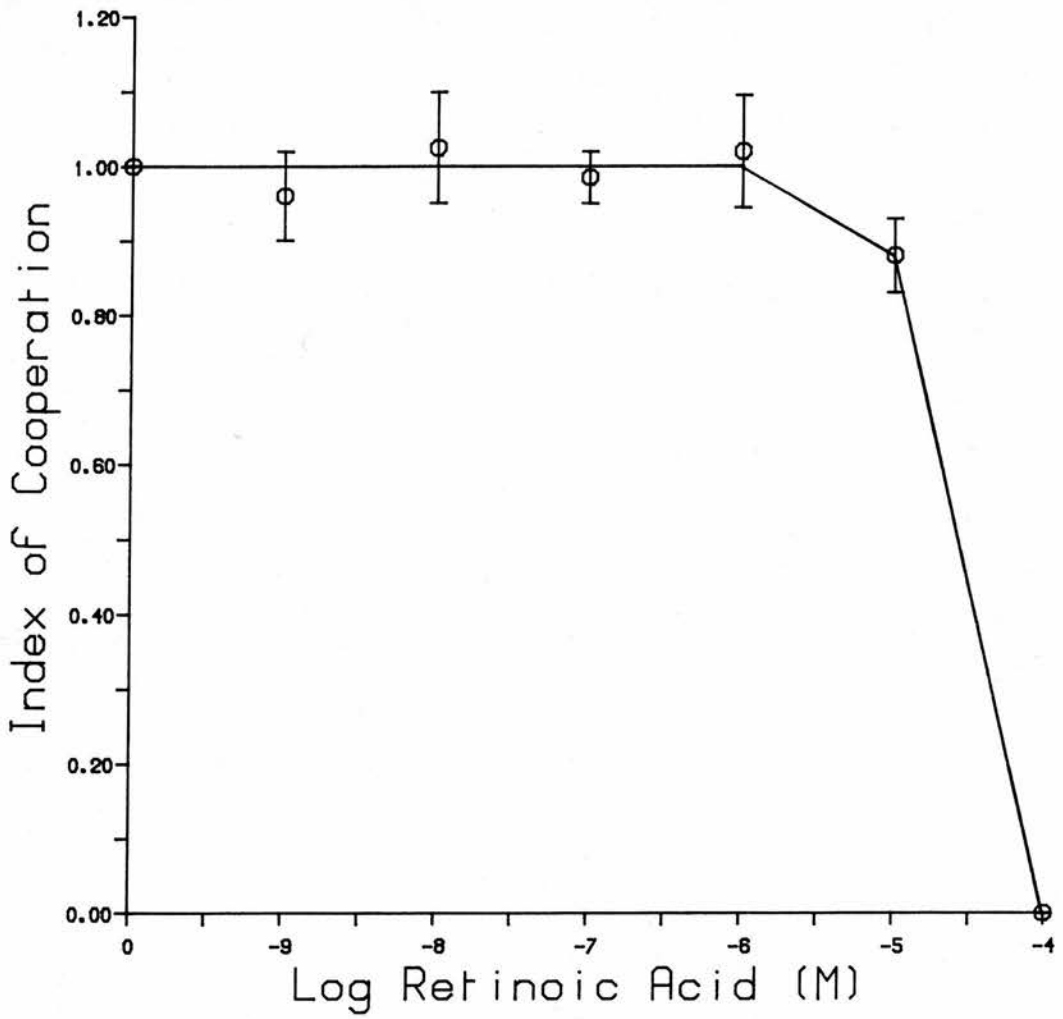
Conc. of Retinoic Acid	Colonies in the Absence of Ouabain		Colonies in the Presence of Ouabain		Index of Cooperation~	
	Expt.1	Expt.2	Expt.1	Expt.2	Expt.1	Expt.2
Control*	176.5	678 (704)	166.5	594 (701)	1.00	1.00
10^{-9} M	168	712	161	563	1.02	0.90
10^{-8} M	182	680	162	660	0.95	1.10
10^{-7} M	158.5	712.5	141	641	0.95	1.02
10^{-6} M	146.5	664.5	130	645	0.95	1.10
10^{-5} M	109	630	85	519	0.83	0.93
10^{-4} M	23	285	0	0	0.00	0.00

*CM containing 0.012M DMSO (0.04M DMSO for figures in parentheses)
~Defined in Section 2.11.
Assays were performed as detailed in Section 2.11. Data are the means of duplicate determinations.

Fig.4.1 Ouabain Rescue Assays on PC13.5 in the Presence of Retinoic Acid

Ouabain rescue assays were performed in duplicate according to the modified procedure described in Section 2.11. Each data point is the mean±1/2 range determined from two independent experiments.

Fig. 4.1 Ouabain Rescue Assays on PC13.5
in the Presence of Retinoic Acid



No colonies grew up in the presence of ouabain in control cultures established on gelatinized dishes in the absence of STO feeders. Differentiation is therefore not accompanied by the acquisition of ouabain resistance and this does not underlie the survival of colonies on feeder layers. Control cultures in gelatinized dishes contained some morphologically differentiatied cells after exposure to 10^{-9}M retinoic acid, and their proportion increased with increasing retinoid concentration until over 90% of cells were overtly differentiated at 10^{-6}–10^{-5}M. On feeder layers differentiated cells of probable teratocarcinoma origin became apparent on the periphery of some PC13.5 colonies in 10^{-7}M retinoic acid. Small numbers of such cells may have been present at lower concentrations, but not distinguished from the feeder layer background. Even at 10^{-7}M retinoic acid, the nature of the differentiated cells cannot be conclusively established by microscopic examination. At higher concentrations, however, differentiated PC13.5 derivatives are clearly present, as many of the colonies are interspersed mixtures of cell types. In 10^{-4}M retinoic acid, wholly differentiated colonies predominate. These can readily be discriminated from the feeder layer background due to the smaller size, regular morphology and more intense staining of the teratocarcinoma-derived cells.

Consequently, it may be concluded that, within the limits of detection of this assay, retinoic acid at concentrations below 10^{-5}M has no effect on junctional transfer of ions between STO feeder cells and PC13.5 EC cells. At 10^{-5}M its inhibitory effects are marginal, but at 10^{-4}M retinoic acid completely blocks metabolic cooperation. These results are in excellent agreement with those obtained by uridine nucleotide transfer assays on embryonal carcinoma cells (Smith, 1984; Smith *et al*, submitted) and demonstrate that retinoic acid can inhibit junctional transfer of ions aswell as nucleotides and relatively large dye molecules. More importantly, however, they indicate that prolonged exposure of EC cells to retinoic acid does not enhance the inhibitory effect on junctional communication.

Thus retinoic acid exerts an inhibitory action on metabolic cooperation only at concentrations four orders of magnitude greater than required for induction of differentiation in the absence of feeders and at least one order of magnitude greater than required for induction of differentiation in the presence of feeders.

4.3. THE ISOLATION AND CHARACTERISATION OF DIFFERENTIATION-DEFICIENT EC CELLS

Feeder-independent PC13.5 EC cells (Hooper and Slack, 1977) were used as the starting material for this selection as they show negligible levels of spontaneous differentiation but differentiate readily on exposure to retinoic acid (Adamson *et al*, 1979). Moreover, the differentiation of these cells has been extensively studied (Adamson *et al*, 1979; Rees *et al*, 1979; Heath *et al*, 1981; Rayner and Graham; 1982; Adamson, 1982; Mummery *et al*, 1984; Rayner and Pulsford, 1985). Also, these cells have not passed through any previous selection in retinoic acid, unlike the PCC4aza1R parental line employed by Schindler *et al* (1981) which was originally isolated after a prolonged period of culture in retinoic acid (Jetten *et al*, 1979).

4.3.1. Selection Procedure

Selections were performed in parallel on untreated PC13.5 cells and on PC13.5 cells subjected to mutagenesis by exposure to 2.5×10^{-5}M MNNG (Section 2.6.). Cultures were established at a density of 2.5×10^4 cells/cm^2. Retinoic acid was added to a final concentration of 10^{-5}M from a 10^{-2}M stock solution in DMSO (freshly prepared every 14 days and stored in the dark at -20°C). Trials indicated that differentiation was extensive under these conditions, but once the cultures became confluent residual EC-like cells started to grow up. These cells were not intrinsically resistant to the induction of differentiation, since they displayed wild-type differentiation when plated at low density in retinoic acid. Nor was their appearance a consequence of depletion of retinoic acid as the growth of EC-like colonies persisted when fresh retinoid was added. A possible explanation is suggested by the work of Isacke and Deller (1983) and Heath and Isacke (1983) in which evidence is provided that differentiated PC13 derivatives stimulate the growth of EC cells via both contact-dependent and contact-independent mechanisms. The differentiated cells may thus mimic the effect of fibroblast feeder layers and enable proliferation of EC cells even in the presence of retinoic acid (Ogiso *et al*, 1982; Smith and Hooper, 1983; Rosenstraus *et al*, 1984). To overcome this problem of non-specific growth of EC cells at high density, cultures were trypsinized before reaching confluence (every 3-4 days) and one fifth of the cells were passaged into an equivalent

culture surface area, with the remainder being discarded. Retinoic acid (10^{-5}M) was present throughout the selection. To monitor the efficiency of the selection and facilitate isolation of non-differentiating colonies, plating tests were set up at intervals in which 10^3 cells were inoculated per 100mm dish. These dishes were maintained for 2-3 weeks in the presence of 10^{-5}M retinoic acid, then scored for both total number of colonies of more than 10 cells and number of EC-like colonies. In some cases the latter were individually picked using Pasteur pipettes and transferred to 16mm Linbro wells. Those that continued to proliferate in 10^{-5}M retinoic acid without differentiating were expanded into stock populations referred to as selection intermediates. Cell lines were subsequently established by dilution cloning (Section 2.3.4.) of these intermediates in the continuous presence of 10^{-5}M retinoic acid. The results of the selection are summarized in Table 4.2.

Table 4.2
Results of Plating Tests During the Selection of Differentiation-Defective Variants of PC13.5

a) Non-mutagenized PC13.5 starting material

Rounds of Selection	Total Colonies	EC-like Colonies	Intermediate	Clones
0	1	0	-	-
3	2	0	-	-
6	10	2	PRA0	PRA0.1,PRA0.4

b) Mutagenized PC13.5 starting material

Rounds of Selection	Total Colonies	EC-like Colonies	Intermediate	Clones
0	1	0	-	-
3	9	3	PRA3*	-
6	6	2	-	-
7	11	5	PRA7	PRA7a,PRA7b,Pra7c

*Lost due to microbial contamination

For details see text

Relative to PC13.5, all the clones showed reduced differentiation after exposure to 10^{-5}M retinoic acid as assessed by the fibrin overlay assay for plasminogen activator production (Section 2.10.5.). Two clones, PRA0.1 derived from non-mutagenized PC13.5, and PRA7c, derived from MNNG-treated PC13.5, were taken for further study as they scored lowest in the fibrin overlay assays and displayed the same karyotype as PC13.5: a modal chromosome number of 41, with 1 metacentric marker chromosome.

4.3.2. Properties of The Differentiation-Deficient Clones PRA0.1 and PRA7c

PRA7c cells (Plate 4.1F) are morphologically indistinguishable from PC13.5 cells (Plate 4.1A), growing predominantly as packed epithelial clusters, even at low density. PRA0.1 cells (Plate 4.1C) at low density appear rather larger and more spread than PC13.5, but similar morphology is exhibited by other clones of PC13 such as PC13TG8 (Hooper and Slack, 1977). As they near confluence, PRA0.1 monolayers become more densely packed and the cells assume typical embryonal carcinoma morphology.

Like other EC cells and in contrast to differentiated teratocarcinoma cells, the growth of PRA0.1 and PRA7c is not subject to contact inhibition. Consequently their maintenance demands continual subculturing.

In common with other EC cells, neither PRA0.1 nor PRA7c produce significant amounts of plasminogen activator (Table 4.3) in the absence of retinoic acid.

The indirect immunoperoxidase technique (Section 2.19.) was used to investigate the expression of two cell surface antigens characteristic of inner cell masses of preimplantation mouse embryos and of embryonal carcinoma cells. The mouse IgM monoclonal antibody against stage-specific embryonic antigen-1 (SSEA-1) reacts only with embryonal carcinoma cells and preimplantation mouse embryos (Solter and Knowles, 1978). The rat IgM monoclonal antibody M1/22-25 (Stern *et al*, 1978) recognises a Forssmann specificity present on teratocarcinoma stem cells but absent from other tumour cells and from many differentiated cell types. Over 75% of cells in PC13.5, PRA0.1 or PRA7c monolayers fixed with acetone displayed a positive immunoperoxidase reaction on their cell surface following incubation with anti-SSEA-1 (Table 4.4). Approximately 40% of cells of all three lines specifically bound M1/22-25. No specific staining was produced when normal rabbit serum was employed as the primary layer. An inability to label all cells in morphologically homogeneous EC populations has previously been reported for M1/22-25 and other antisera to embryonal carcinoma (Stern *et al*, 1975; Stern *et al*, 1978) and may reflect variations in antigen availability during the cell cycle.

In the absence of retinoic acid PRA0.1 and PRA7c exhibit the characteristics of PC13.5 and other EC cells in their morphology, growth behaviour, production of

plasminogen activator, and expression of SSEA-1 and Forssman antigen (Table 4.4).

Table 4.3
Fibrin Overlay Assays For Plasminogen Activator Production By PC13.5, PRA0.1 and PRA7c

Cell Line	Retinoic Acid	Expt.1 C	Expt.1 L	Expt.2 C	Expt.2 L	Expt.3 C	Expt.3 L	% Positive Colonies*
PC13.5	-	638	25	318	6	505	4	2.2±0.9
	+	534	455	310	276	358	331	87.5±3.3
PRA0.1	-	297	4	361	7	442	0	1.1±0.6
	+	302	49	426	90	359	21	14.5±4.4
PRA7c	-	254	15	232	32	129	0	6.6±4.0
	+	234	2	211	25	148	0	4.3±3.8

*Mean ± s.e.m.

Cultures were incubated for 5 days in 10^{-5}M retinoic acid. Fibrin overlays were allowed to develop for 4 hours then scored for total number of colonies (C) and number of lytic zones (L).

Table 4.4
Effects of 10^{-5}M Retinoic Acid on PC13.5, PRA0.1 and PRA7c

Cell Line	RA*	Morphology^	Growth Capacity	Antigen Expression SSEA-1	Forssman	Plasminogen Activator
PC13.5	-	EC	Unlimited	++	+	-
	+	END	Limited	-	++	+
PRA0.1	-	EC	Unlimited	++	+	-
	+	EC	Unlimited	+	+	±
PRA7c	-	EC	Unlimited	++	+	-
	+	EC	Unlimited	++	+	-

*Presence (+) or absence (-) of 10^{-5}M retinoic acid for 5 day culture period.
^EC, embryonal carcinoma
END, differentiated (Adamson *et al*, 1979).
Results of specific immunoperoxidase staining of cells or of plasminogen activator production by colonies are recorded as follows:-

- less than 10% positive
± 10-20% positive
+ 30-60% positive
++ more than 75% positive

When plated at $4x10^3$ cells per cm^2 and cultured in the presence of 10^{-5}M retinoic acid, PC13.5 forms monolayers which consist almost entirely of differentiated flattened cells (Plate 4.1B). Growth of these cultures does not persist after subculturing, even if retinoic acid is no longer present. The

growth and morphology of PRA0.1 and PRA7c in 10^{-5}M retinoic acid is in marked contrast to the above. PRA0.1 cells initially undergo morphological changes characteristic of the first 48 hours of the response of PC13.5 to retinoic acid. The cells move apart and spread out over the substratum, becoming less regular in shape. These changes do not culminate in overt differentiation of the bulk population, however. Instead the culture develops into a mixture of clusters of EC-like cells interspersed with cells of intermediate morphology (Plate 4.1D) and the occasional island of overtly differentiated cells. Even in the continuous presence of 10^{-5}M retinoic acid, the EC cells progressively overgrow the other cell types so that as the culture nears confluence it is composed predominantly of embryonal carcinoma (Plate 4.1E). On subculturing the same cycle of changes is repeated and has been found to occur for at least 5 serial passages in 10^{-5}M retinoic acid. Regeneration of EC cells may arise from a fraction of the population which does not undergo the initial transition to an intermediate morphology. Alternatively, the latter may represent a reversible stage in the differentiation process which is capable of dedifferentiation to the EC phenotype. Ogiso *et al* (1982) have provided evidence for reversibility in the early stages of F9 embryonal carcinoma cell differentiation. On removal of retinoic acid the culture assumes the characteristics of an untreated PRA0.1 culture after a further subculture. PRA7c cells have also been maintained for 5 serial passages in 10^{-5}M retinoic acid. They retain typical EC cell morphology throughout (Plate 4.1G,H) and remain insensitive to density-dependent growth inhibition.

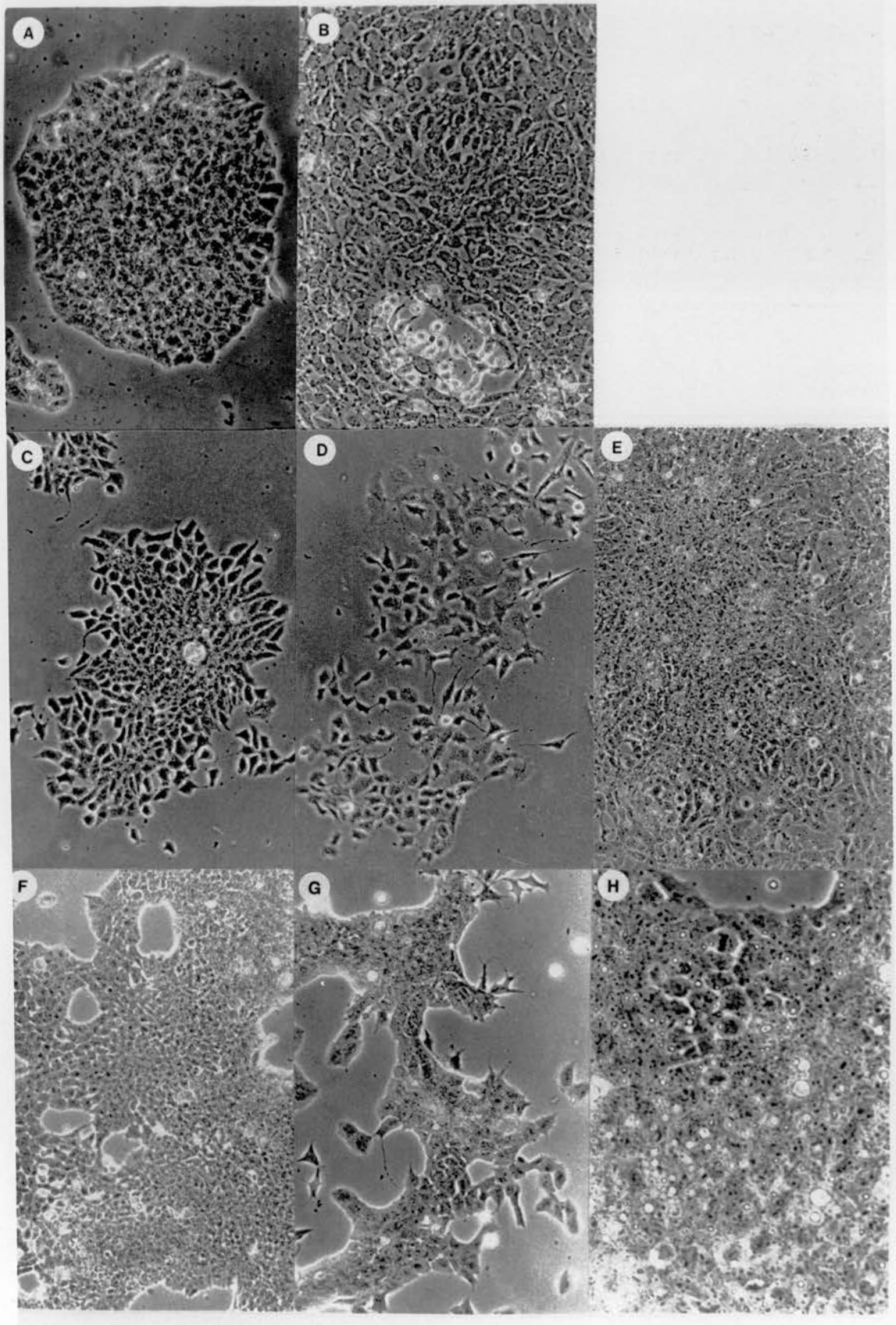
Differentiation of PC13.5 in retinoic acid is accompanied by loss of SSEA-1 expression. After 4 days in 10^{-5}M retinoic acid less than 10% of cells show a positive indirect immunoperoxidase reaction. Forssman antigen apparently continues to be expressed by the differentiated cells, however, as most of the population gives a positive reaction with M1/22-25. PRA0.1 and PRA7c continue to express SSEA-1 as well as Forssman antigen after 4 days in retinoic acid. Similar high levels of indirect immunoperoxidase staining for SSEA-1 are found in retinoic acid-treated and untreated populations of both cell lines.

The fibrin overlay assay for plasminogen activator production was used to obtain a semi-quantitative estimate of the differentiation responses of PRA0.1 and PRA7c to 10^{-5}M retinoic acid. The data listed in Table 4.3 show that

Plate 4.1 PC13.5, PRA0.1 and PRA7c in the Presence and Absence of 10^{-5}M Retinoic Acid

Phase contrast

	Magnification
A. PC13.5	276
B. PC13.5 plus retinoic acid, 7 days	108
C. PRA0.1	108
D. PRA0.1 plus retinoic acid, 3 days	86
E. PRA0.1 plus retinoic acid, 7 days	86
F. PRA7c	86
G. PRA7c plus retinoic acid, 5 days	108
H. PRA7c plus retinoic acid, 7 days	276


A
B
C
D
E
F
G
H

whereas nearly 90% of PC13.5 cells give rise to plasminogen activator producing derivatives within 5 days, this is true for less than 20% of PRA0.1 cells. The proportion of plasminogen activator-positive PRA7c colonies is not significantly affected by incubation with retinoic acid. This biochemical index of differentiation is therefore in agreement with evidence provided by cell morphology, growth behaviour and SSEA-1 expression, that PRA0.1 has a greatly reduced differentiation response to retinoic acid and PRA7c is apparently completely resistant to the induction of differentiation by retinoic acid. These results are summarised in Table 4.4.

To investigate the tumourigenicity of the clones and their developmental capacity *in vivo*, $2x10^6$ cells were injected subcutaneously into individual mice of strain 129. Two mice were injected with PRA0.1 and both developed palpable tumours at the site of injection within 5 weeks. These contained areas of considerable necrosis with the surviving tissue consisting of mitotically active undifferentiated embryonal carcinoma and stroma. The latter was probably of host origin. PC13.5 tumours in contrast generally contain considerable amounts of neurepithelium and nervous tissue in addition to embryonal carcinoma, with smaller contributions from cartilage and various other epithelia (Hooper and Slack, 1977). Subject to confirmation by analysis of more tumours, PRA0.1, whilst retaining tumourigenicity, appears to have lost the capacity for differentiation *in vivo*. Three mice injected with PRA7c all failed to produce tumours within nine months, indicating that this clone may not be tumourigenic. This conclusion is also subject to confirmation by injection of further mice. Loss of tumourigenicity is relatively common following mutagenesis of EC cells and is not necessarily associated with loss of differentiation potential (Boon *et al*, 1974).

The junctional communication properties of PRA0.1 and PRA7c were examined using the standard ouabain rescue protocol (Section 2.11.). Both clones are ouabain sensitive and produced no colonies when exposed to ouabain in the absence of a feeder layer. Relatively high numbers of colonies of both lines did grow up in ouabain in the presence of feeders (Table 4.5), however, indicating that the cells undergo metabolic cooperation for ions with STO fibroblasts. The communication ability of PRA0.1 and PRA7c in this assay is comparable to that of PC13.5, as reflected in the values for the index of cooperation. The cooperation-defective PC13 variant R5/3 (Slack *et al*, 1978) was included as a negative control in these experiments and as expected

showed a low index of cooperation relative to PC13.5.

Table 4.5
Ouabain Rescue Assays on PC13.5, PRA0.1, PRA7c and R5/3

Cell Line	Colonies in the Absence of Ouabain*	Colonies in the Presence of Ouabain*	Index of Cooperation^
PC13.5	750	663	1.00
PRA0.1	810	788	1.10
PRA7c	868	731	0.95
R5/3	370	69	0.21

*Mean of three determinations
^Defined in Section 2.11.

Normal junctional communication properties are also exhibited by the differentiation-deficient EC clones isolated by McBurney and co-workers (Campione-Piccardo *et al*, 1985).

4.4. DETECTION OF CRABP IN EC CELL CYTOSOLS

The simplified procedure described in Section 2.12. was employed to screen cytosols prepared from various EC cell lines for the presence of retinoic acid-binding activity. Instead of sucrose gradient analyses, the [^{3}H]-retinoic acid bound in triplicate assays was quantified by liquid scintillation counting directly after extraction of unbound retinoid with charcoal/dextran. A representative result for such an experiment is detailed in Table 4.6. The teratocarcinoma-derived parietal endoderm line PYS-2 (Lehman *et al*, 1974) which has previously been reported to lack CRABP (Matthaei *et al*, 1983) does not show any component of binding displaceable by excess unlabelled retinoic acid. In contrast the amount of [^{3}H]-retinoic acid bound by PTmr0 cytosols which do possess CRABP (Section 3.3.1.) is significantly reduced by excess unlabelled retinoic acid, though not by retinol or retinal. Such saturable and specific binding is presumed to arise from CRABP as sucrose gradient analyses indicate that this is the only entity in EC cell cytosols which binds significant amounts of retinoic acid (Fig.3.3; see also Sherman *et al*, 1983).

This assay protocol has proved qualitatively reproducible but quantitative reproducibility has been variable. The data from a series of such assays are summarised in Table 4.7 in terms of the presence (+) or absence (-) of

displaceable binding (CRABP). A positive result is recorded where the c.p.m. retained in the absence of unlabelled retinoic acid is significantly greater (*t* test, $P<0.001$) than the c.p.m. retained in the presence of a 200-fold excess of unlabelled retinoic acid. Comparisons with sucrose gradient analyses are included where applicable.

Table 4.6
Exchange Assays for Retinoic Acid-Binding Activity in Cytosols of PYS and PTmr0

Cell Line	Addition	Total c.p.m.*	Specific c.p.m.
PYS	Retinoic acid	12,859 ± 298	-
	None	11,173 ± 124	0
PTmr0	Retinoic acid	6,430 ± 103	-
	None	9,217 ± 141	2,787
	Retinol	9,126 ± 49	2,696
	Retinal	9,358 ± 139	2,928

*Mean c.p.m. ± s.e.m. for triplicate determinations

Table 4.7
Detection of CRABP in Cytosols by Exchange Assays and by the Sucrose Gradient Procedure

Cell Line	Exchange*	Sucrose Gradient^
PYS	- (3)	-
PSA4TG12[a]	+ (1)	+
PTmr0[a]	+ (1)	+
PT2[b]	+ (2)	N.D.
PT2md1	+ (2)	N.D.
PC13.5	+ (1)	+
PRA0.1	+ (2)	N.D.
PRA7a	+ (2)	+
PRA7c	+ (2)	N.D.

*Figures in parentheses indicate the numbers of separate cytosols assayed.
^N.D.=not determined
[a] Grown in feeder-conditioned medium
[b] Grown on STO feeders

For details see text

All the embryonal carcinoma cell lines examined contain CRABP activity, including the differentiation-defective clones PRA0.1, PRA7a, PRA7c (Section 4.3.) and PT2md1 (Section 4.5.).

4.5. EFFECTS OF RETINOIC ACID ON THE VARIANT EMBRYONAL CARCINOMA CLONE PT2md1

4.5.1. Introduction

PT2md1 is a derivative of the EC line PSA4TG12 (Slack *et al*, 1977) isolated by Smith (1984) using the thioguanine 'kiss of death' procedure (Section 1.8). It displays the greatest reduction in metabolic cooperation of a series of such variants. It also shows the poorest differentiation in embryoid bodies. These two alterations in phenotype may be causally related or merely coincidental (for discussion see Smith, 1984).

Aggregates formed from PT2md1 neither delaminate endoderm nor subsequently develop into cystic embryoid bodies as occurs with PSA4TG12 and its subclone PT2 (Smith, 1984). Instead the vast majority of the aggregates persist as morphologically homogeneous clumps of EC cells. PT2md1 is thus a differentiation-defective EC clone. The nature of the signals which induce differentiation of EC cells in aggregates is poorly understood, as is their relationship to other stimuli for differentiation such as plating in the absence of a feeder layer or exposure to chemical inducers (see Sections 1.4. and 1.5.). The response of PT2md1 to two stimuli which promote differentiation of other PSA4-derived EC cells, namely plating at low density on gelatinised substrata and exposure to retinoic acid, has therefore been investigated. In view of the postulated involvement of retinoid binding proteins in the induction of differentiation (Jetten and Jetten, 1979; Sherman *et al*, 1983; see also Section 1.5.2.) it was considered of interest to determine whether PT2md1 expressed CRABP activity.

4.5.2. Initial Observations

Feeder-dependent EC cells such as PSA4 and its derivatives undergo spontaneous differentiation when plated in the absence of a feeder layer, provided the medium is supplemented with 2-mercaptoethanol (Smith and Hooper, 1983). When seeded at densities of $4x10^3$ cells/cm^2 or less the great majority of the population differentiates within one week and the cells do not proliferate after subculturing. PT2md1 cells in contrast, grow as relatively

homogeneous monolayers of embryonal carcinoma in the absence of feeders, even after seeding at low density (Plate 4.2). Less than 10% of the cells in such cultures display any morphological indications of differentiation. Despite a relatively high level of cell death, feeder-free cultures of PT2md1 have been maintained for over a month (6 passages) during which time there was no apparent change in the growth rate nor any increase in the level of spontaneous differentiation. The continued survival and proliferation of the cells is acutely sensitive to the culture conditions, however. They grow poorly in CM containing foetal bovine serum rather than calf serum, unlike other EC lines grown in this laboratory. Consequently the observations reported below were made in the presence of 10% (v/v) newborn calf serum. Also, as PT2md1 monlayers near confluence they become more dependent than other EC lines on regular changing of the medium. This may arise from their diminished capacity for metabolic cooperation rendering them highly susceptible to the consequences of depletion of nutrients from, or acidification of, the culture medium.

PT2md1 forms colonies with an efficiency of around 15% in the absence of feeders, comparable to its parental line PT2 (Smith, 1984), a subclone of PSA4TG12. Six days after plating at cloning density on gelatinised substrata over 95% of PT2 colonies consist wholly or largely of morphologically differentiated cells. Under the same conditions more than 70% of PT2md1 colonies are composed exclusively of embryonal carcinoma cells when examined by light microscopy (Plate 4.2). PT2md1 thus shows a similar large reduction in its differentiation response to plating in the absence of feeders as it does to aggregation.

Mass cultures of PT2md1 exposed to 10^{-7}M retinoic acid according to the protocol described in Section 2.10.1. display very high levels of cell death and the population does not increase in size after the first 24-48 hours. Most of the surviving cells retain embryonal carcinoma morphology (Plate 4.3B,C), however, and normal growth is restored on removal of the retinoid. These observations contrast with the response of PT2 which, in addition to some cell death, undergoes extensive and irreversible differentiation on exposure to retinoic acid (Plate 4.3A).

Attempts to quantify the differentiation response of PT2md1 to retinoic acid using the fibrin overlay assay for plasminogen activator (Section 2.10.5.) proved

Plate 4.2 PT2mdl Cultured in the Absence of Feeders

Phase contrast
A. Magnification: 200
B. Magnification: 480

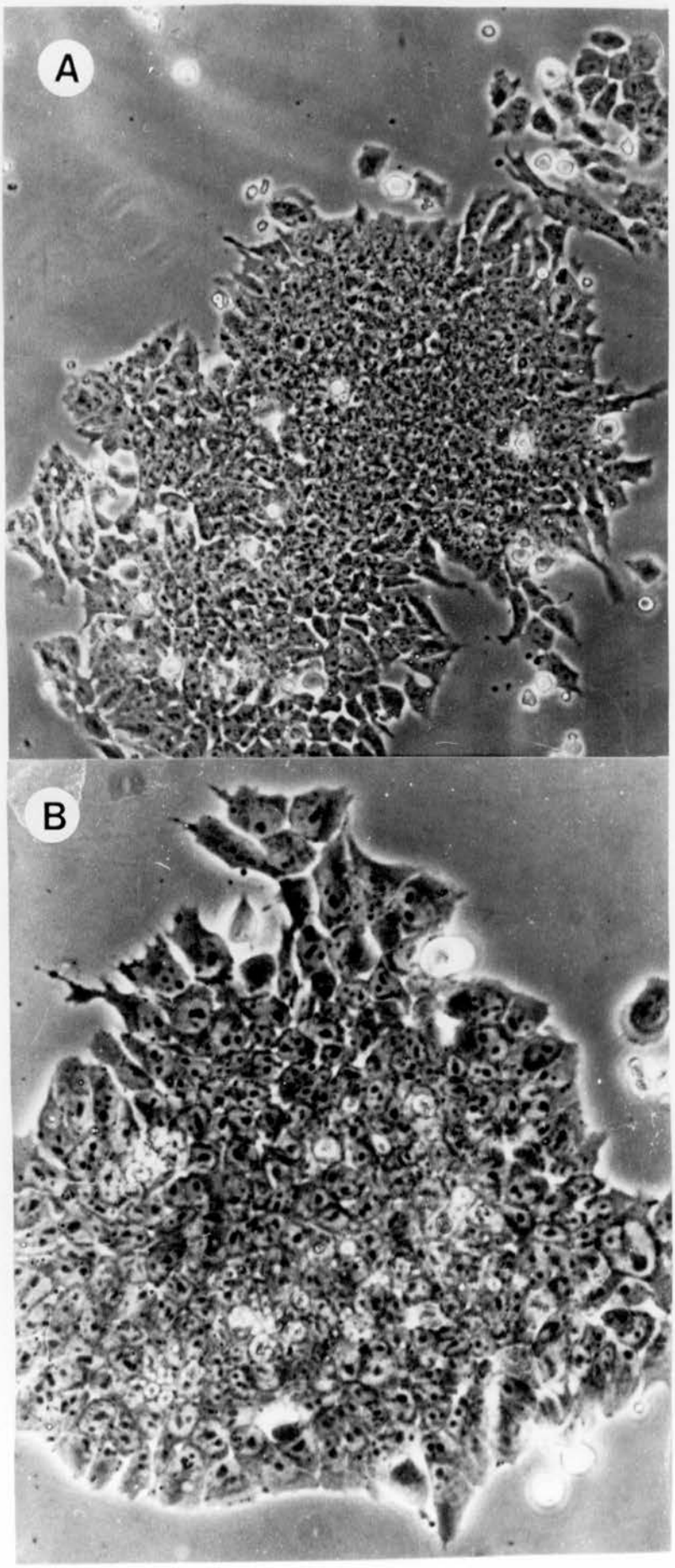
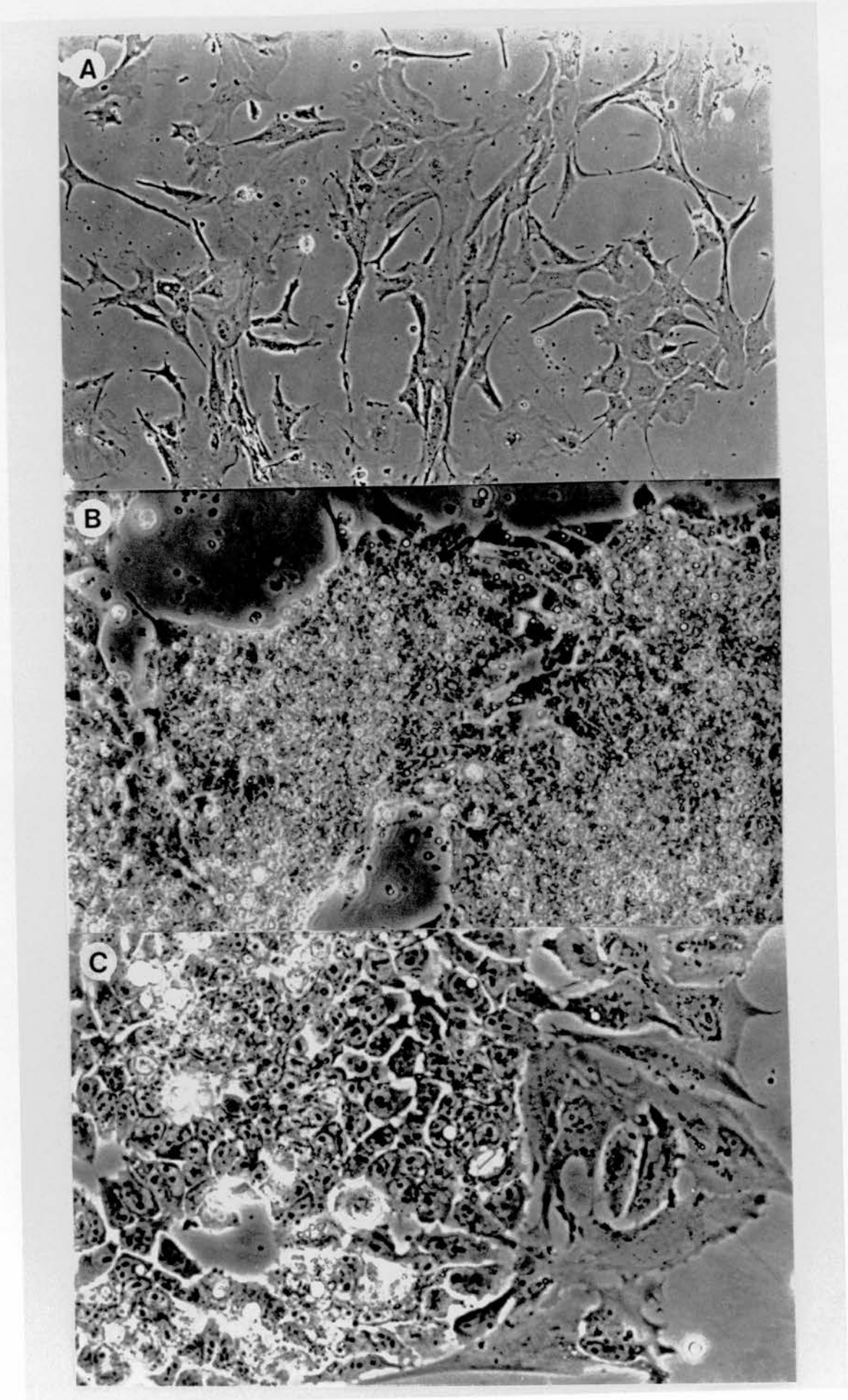

A
B

Plate 4.3 PT2 and PT2md1 in the Presence of 10^{-7}M Retinoic Acid

Phase contrast

	Magnification
A. PT2 plus retinoic acid, 4 days, followed by 3 days in the absence of retinoic acid	124
B. PT2md1 plus retinoic acid, 3 days	124
C. PT2md1 plus retinoic acid, 4 days, followed by 3 days in the absence of retinoic acid. To the right of the field are a few large differentiated cells.	396


A
B
C

unsuccessful as very few colonies survived incubation with 10^{-7}M retinoid (see following Section). Notwithstanding this lack of quantification, it seems reasonable to conclude that PT2md1 shows little if any induction of differentiation by retinoic acid.

Cytosols prepared from log phase feeder-free cultures of PT2md1 displayed a high affinity saturable component of [^{3}H]-retinoic acid binding ascribed to CRABP (Table 4.7). Therefore neither the differentiation-defective phenotype, nor the heightened mortality in retinoic acid described in the following Section, arise from lack of this binding protein.

4.5.3. Toxicity of retinoic acid to PT2md1

Following the finding that 10^{-7}M retinoic acid was highly toxic to PT2md1, toxicity tests were carried out on PT2 and PT2md1 over the concentration range 10^{-9}-10^{-5}M. The results of these experiments are illustrated in Fig.4.2. The number of colonies formed per 1000 cells seeded by both cell lines in the presence of 0.012M DMSO (employed as solvent for the retinoic acid) was between 100 and 200. There is a marked difference in the susceptibility of the two lines to the toxic effects of retinoic acid, with PT2 being far more resistant than its differentiation-defective variant. The dramatic drop in PT2md1 colony number between 10^{-9}-10^{-8}M retinoic acid coincides with a decrease in the percentage of PT2 colonies containing EC cells (Fig.4.4). The latter are identified by their small size, tight packing and intense staining with Leishmans'. Approximately one third of colonies contain some EC-like cells 6 days after plating in control CM supplemented with DMSO alone. Retinoic acid at 10^{-9}M reduces this proportion to around 20%, but at 10^{-8}M it is down to less than 5% i.e. 10^{-8}M retinoic acid eliminates EC cells from over 80% of those colonies which retain them in CM alone. This loss of EC cells can be used as a measure of the response to retinoic acid (Table 4.8).

Fig.4.2 Toxicity of Retinoic Acid to PT2 and PT2mdl in CM

Plating tests were performed in triplicate as described in Section 2.9. Each data point is the mean±s.e.m. of four independent experiments.

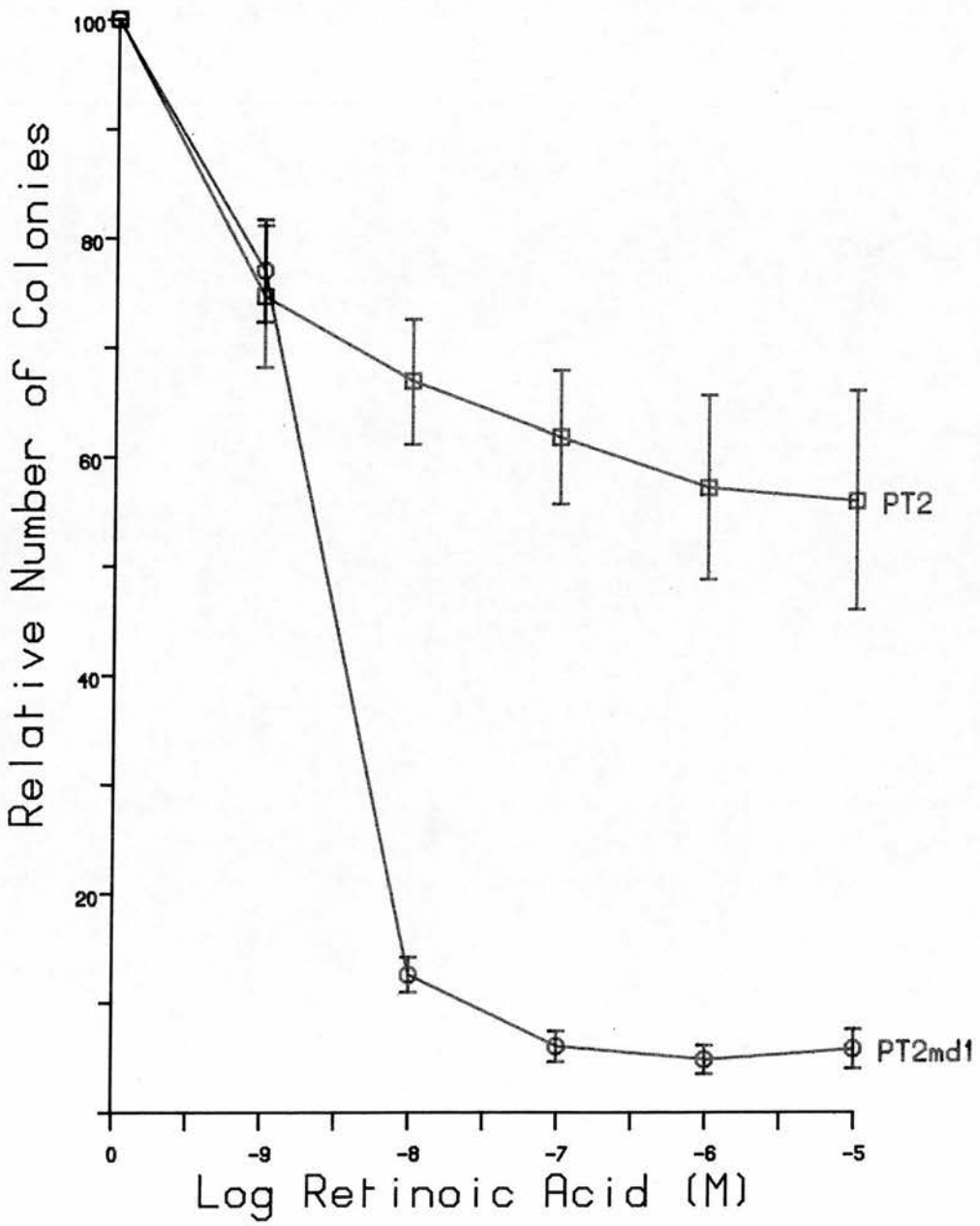

Relative Number of Colonies
100
80
60
40
20
0
-9
-8
-7
-6
-5
Log Retinoic Acid (M)
PT2
PT2md1

Fig.4.3 Toxicity of Retinoic Acid to PT2 and PT2md1 in CM and in Conditioned Medium

Plating tests were performed in triplicate as described in Section 2.9. in either CM and STO feeder-conditioned CM (a) or in CM and BRL-medium (b). Solid lines indicate CM and broken lines conditioned medium. Each data point is the mean±1/2 range of two independent experiments in (a) and mean±s.e.m. of a single experiment in (b).

Fig.4.3 Toxicity of Retinoic Acid to PT2 and PT2md1 in CM or in Conditioned Medium

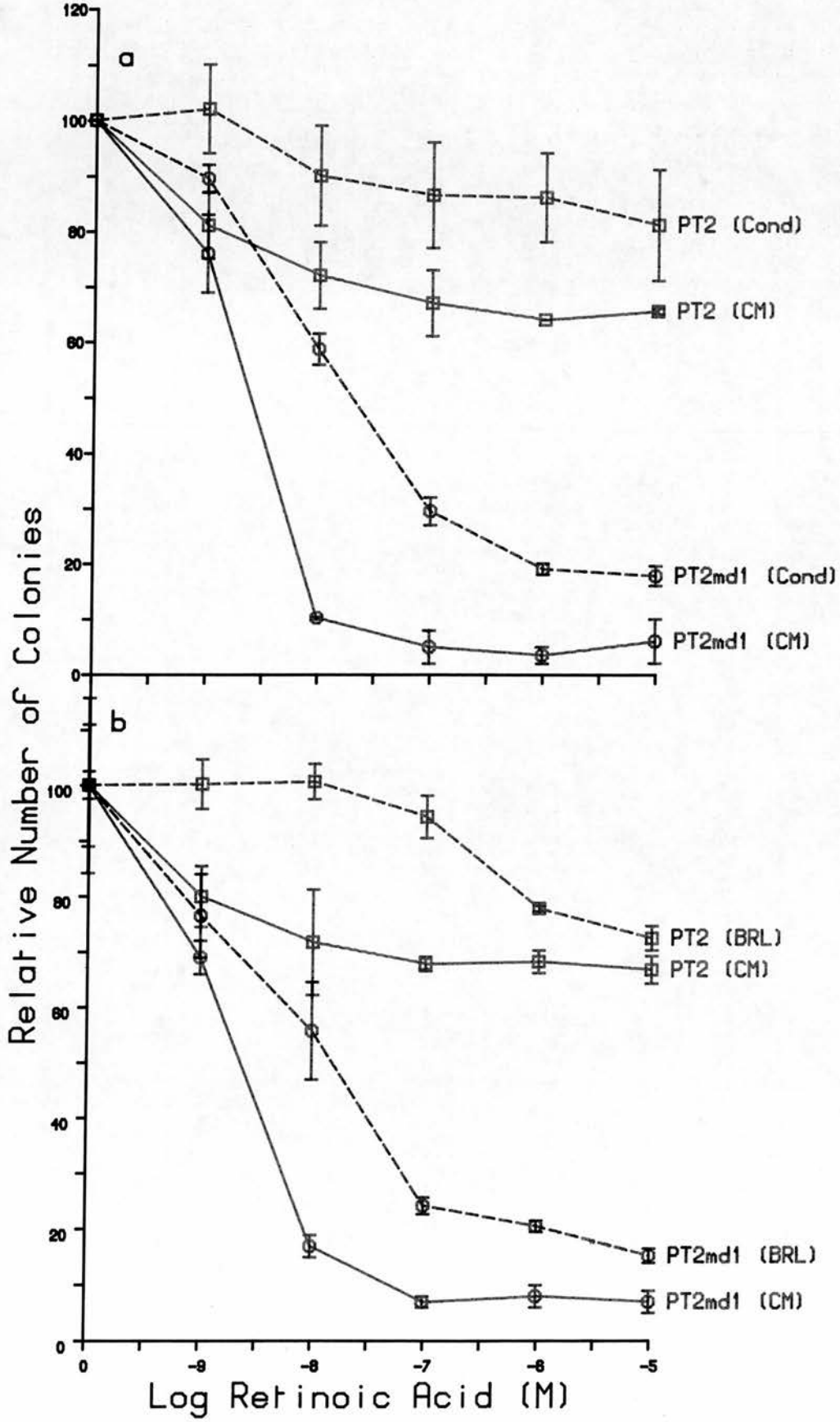


Fig.4.4 Mortality of PT2mdl and Differentiation of PT2mdl Induced by Retinoic Acid

Plating tests were performed in triplicate as describd in Section 2.9. in CM (solid lines) or in feeder-conditioned medium (broken lines). Mortality of PT2mdl is indicated by the red lines. Each data point is the mean±1/2 range of two independent experiments. Induced differentiation of PT2, as estimated by loss of EC cells (Table 4.8) is indicated by the blue lines.

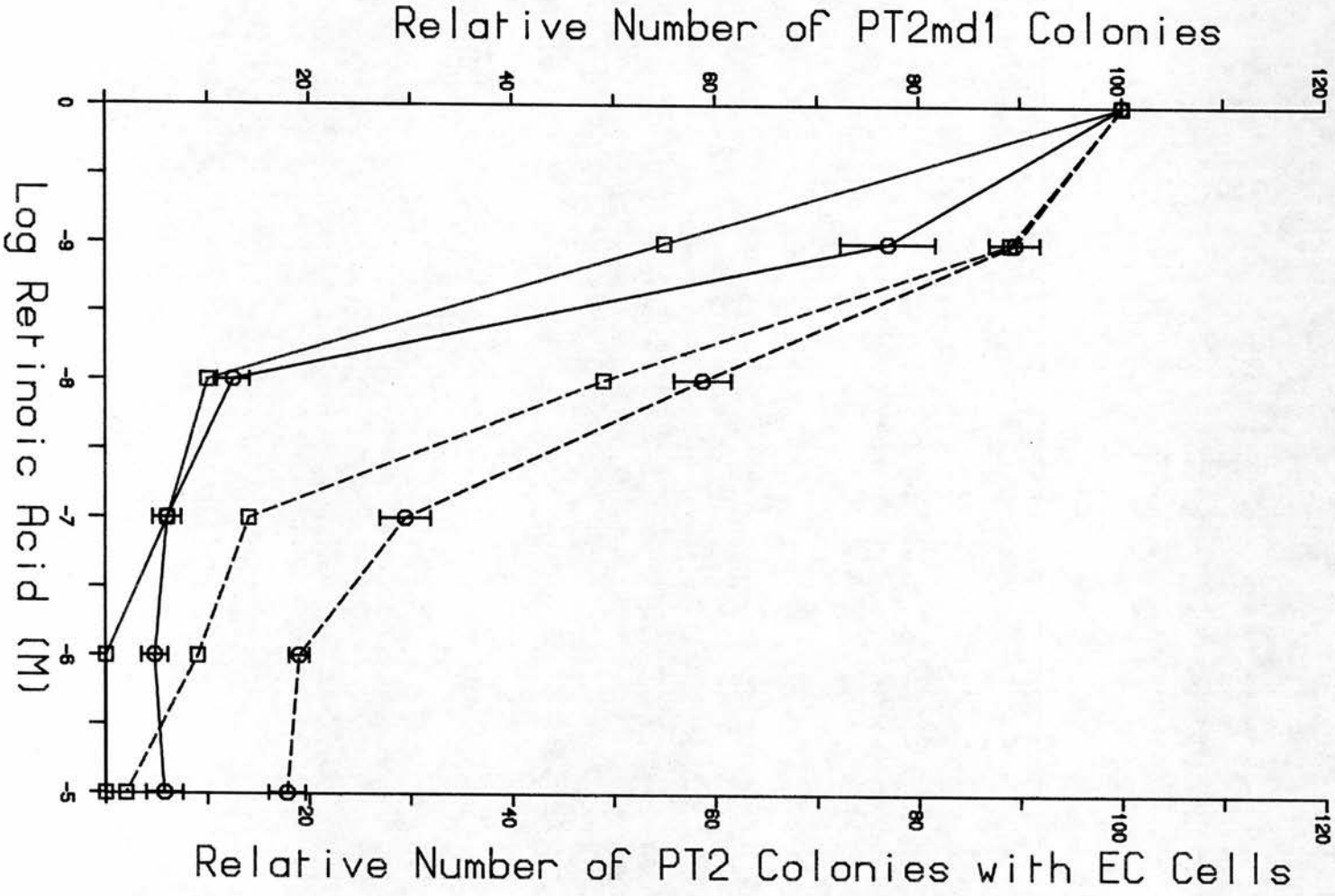

Relative Number of PT2md1 Colonies
20
40
60
80
100
120
0
-9
-8
-7
-6
-5
Log Retinoic Acid (M)
Relative Number of PT2 Colonies with EC Cells

Table 4.8
The Induction of EC Cell Loss from PT2 Colonies by Retinoic Acid*

Concentration of Retinoic Acid	EC Cells in CM	EC Cells in Feeder-Conditioned CM
Control	100(34)^	100(75)^
10^{-9}M	55	89
10^{-8}M	10	49
10^{-7}M	6	14
10^{-6}M	0	9
10^{-5}M	0	2

*Expressed as a percentage of the number of colonies containing residual EC cells in the presence of DMSO alone.
^Figures in parentheses denote percentage of total colonies containing EC cells.

Data are the means of two experiments fixed and stained with Leishmans after 6 days in retinoic acid and individually inspected for the presence of EC cells.

Neither PT2 nor PT2md1 were exposed to retinoic acid during their isolation and there is no *a priori* explanation for their different sensitivities. The possibility presented itself, however, that the susceptibility of PT2md1 to retinoic acid toxicity might be linked to its restricted capacity for differentiation. Medium conditioned by STO feeder layers has been shown to cause partial inhibition of both the spontaneous differentiation of feeder-dependent embryonal carcinoma cells plated on gelatinized substrata and the retinoic acid-induced differentiation of feeder-independent EC lines (Smith and Hooper, 1983). Medium conditioned by Buffalo rat liver (BRL) cells contains a more potent "differentiation inhibiting activity" to be described in Chapter 5. The results of retinoic acid toxicity tests on PT2 and PT2md1 in 100% (v/v) feeder-conditioned CM and in 60% (v/v) BRL-conditioned medium (BRL-medium) are depicted in Figs.4.3a and 4.3b respectively.

The conditioned media increase the resistance of both cell lines to the toxic effects of retinoic acid. They also reduce the extent of differentiation of PT2 both in control cultures supplemented with DMSO alone and in the presence of retinoic acid. In unmodified and feeder-conditioned medium where there is a high level of spontaneous differentiation it is difficult to quantify any specific responses to retinoic acid (Section 3.2.1.). However, some indication of the responsiveness of PT2 cells to retinoic acid is provided by the loss of residual

EC cells from colonies. This loss seems to be due, at least in part, to an induction of differentiation (see discussion in following Section). The data in Table 4.8. may therefore be considered as an index, admittedly rather crude, of the differentiation response of PT2.

It is not appropriate to apply the same measure to cultures grown in BRL-medium, however. The level of spontaneous differentiation in this medium is negligible (Chapter 5). Consequently EC cells persist in the majority of colonies until the retinoic acid concentration exceeds 10^{-6}M. Such a difference in the overall degree of differentiation does not, however, imply any difference in the specific response to retinoic acid. One index of the latter in BRL-medium is the production of plasminogen activator. This cannot be directly compared with EC cell loss in unmodified or feeder-conditioned medium. Nonetheless, the results contained in Section 3.2.1. demonstrate that the induction of differentiation of PSA4TG12 in BRL-medium by retinoic acid is concentration-dependent. Morphological observations on PT2 indicate that it responds in a similar fashion to its parental line on exposure to retinoic acid; the proportions, both of colonies containing differentiated cells and of differentiated cells within colonies, increase with increasing retinoic acid concentration.

To further investigate the possibility of a relationship between cell death and differentiation the effects of two other chemicals on PT2 and PT2md1 were studied. Retinol is the alcohol form of vitamin A which is identical in structure to retinoic acid apart from replacement of the terminal carboxyl group with an alcohol group (see Section 1.5.). It is a 100-1000 fold less potent inducer of EC cell differentiation than the acid derivative (Sherman *et al*, 1983). Hexamethylenebisacetamide (HMBA) is a non-retinoidal compound which induces differentiation of murine erythroleukaemia cells and some EC cell lines (Jakob *et al*, 1979; Speers *et al*, 1979; McCue *et al*, 1983).

Colony formation by PT2 and PT2md1 in the presence of these two chemicals was investigated (Section 2.9). The results listed in Table 4.9 indicate that HMBA, like retinoic acid, promotes the differentiation of PT2 (see also Plate 4.4B) and is likewise highly toxic to PT2md1. The number of colonies formed by the latter in the presence of 3mM HMBA is less than 20% of the number obtained in CM alone. However, BRL-medium strongly inhibits the induction of differentiation of PT2 by HMBA and in this medium PT2md1 retains a high

efficiency of colony generation.

Table 4.9
Colony Formation By PT2 and PT2md1 in Retinoic Acid, Retinol and HMBA*

Chemical	Expt.	Colony Formation PT2	Colony Formation PT2md1	Differentiation of PT2^
Retinoic	1	75.4	7.5	++
acid (10^{-7} M)	2	67.8	7.0	++
Retinol	1	89.6	78.9	±
(10^{-7} M)	2	66.0	27.0	+
HMBA (3mM)	1	73.5	14.9	++
	2	74.6	19.0	++
HMBA (3mM)	1	ND	ND	ND
in BRL-medium	2	93.8	82.4	-

*Relative to colony formation in unmodified CM or BRL-medium
^Morphological assessments of a minimum of 100 PT2 colonies:-
- >90% consist wholly or largely of EC cells
± 25-35% contain EC cells (equivalent to controls in unmodified CM)
+ <10% contain EC cells
++ <5% contain EC cells
Data are the means of triplicate determinations

The inhibitory actions of BRL-medium on both induction of PT2 differentiation and toxicity to PT2md1 are greater for HMBA (Table 4.9) than for retinoic acid (Fig.4.3b), possibly reflecting some difference in the mode of action of these two inducers. The two experiments with retinol provide contrasting results (Table 4.9). In the first induction of differentiation was slight as assessed by the proportion of colonies retaining EC cells. The number of colonies formed by both cell lines was only slightly reduced in this case. In the second experiment, however, a significant level of differentiation was induced such that the proportion of colonies retaining EC cells decreased from 30% in cultures exposed to DMSO alone to 8% in cultures exposed to 10^{-7}M retinol. This induction of PT2 differentiation was accompanied by an increase in toxicity towards PT2md1.

The different response to retinol in the two experiments may reflect variation in the amount of retinol added some change in the culture conditions, such as a difference in serum retinoid levels or the amount of serum retinol binding protein (RBP), or could be due to oxidation of the retinol to retinoic acid. Whatever the explanation it appears that for retinol as for HMBA and retinoic

acid, conditions which lead to the induction of differentiation of PT2 result in cell death of PT2md1. Conversely conditions which partially or wholly inhibit the induction of differentiation of PT2 similarly reduce toxicity towards PT2md1.

4.5.4. Discussion

PT2md1 exhibits several novel properties in addition to the cooperation-defective phenotype for which it was originally selected. Its restricted potential for differentiation in aggregates has previously been described (Smith, 1984) but it also has a greatly diminished capacity for spontaneous differentiation in monolayer culture. It has thus acquired a quasi-feeder-independent phenotype in that it no longer requires a feeder layer for maintenance of an undifferentiated stem cell population.

Previously characterised feeder-independent EC cell lines have either been obtained from nullipotent tumours (Martin and Evans, 1975b) or been isolated after a period of culture in the absence of feeders (Bernstine *et al*, 1973). PT2md1, in contrast, is derived from a pluripotential EC clone and was isolated on STO feeder layers (Smith, 1984). The thioguanine 'kiss of death' protocol employed to isolate PT2md1 does involve some selection for feeder-independence, however. Firstly, it exerts direct selective pressure for EC cells which do not cooperate, thus selecting against any requirement for junctional communication with the feeders. Secondly, it almost certainly also exerts a degree of selection for independence from diffusible factors produced by the feeders, since thioguanine, via its incorporation into nucleic acids, disrupts protein synthesis by the feeder cells and eventually causes their disintegration. It is conceivable that these factors in the selection process may underlie not only the apparent feeder-independence of PT2md1, i.e. its poor differentiation on gelatinized substrata, but concomitantly could have resulted in its restricted differentiation in embryoid bodies (see also discussion in Smith, 1984).

PT2md1 also differs from other feeder-independent cell lines in its absolute requirement for 2-mercaptoethanol for survival on gelatinised substrata (see Chapter 7). It should be noted in this regard that 2-mercaptoethanol was present throughout the selection procedure.

Unlike most EC cell lines PT2md1 is unresponsive to induction of differentiation

by retinoic acid or HMBA. With the exception of a few clones selected specifically for non-responsiveness to chemical inducers, all other EC lines tested differentiate when exposed to retinoic acid (see Section 4.3.). The caveat should be entered that it has not been rigorously established that retinoic acid stimulates differentiation of feeder-dependent EC lines such as PT2 in unmodified medium (see Section 3.2.1.). However, the results presented in Section 3.2.1., Section 4.4.3. and Section 5.3.6. demonstrate that such cells do differentiate in response to retinoic acid in BRL-medium. It therefore seems likely that the promotion of EC cell disappearance from PT2 cultures in normal medium is due to induction of differentiation rather than to a specific cytotoxic action of the retinoid (though the latter may also operate, see below). The evidence in favour of induction of differentiation is even clearer for HMBA because cells produced after exposure to this chemical differ in morphology from those which have differentiated spontaneously in the same medium (Plate 4.4, see below).

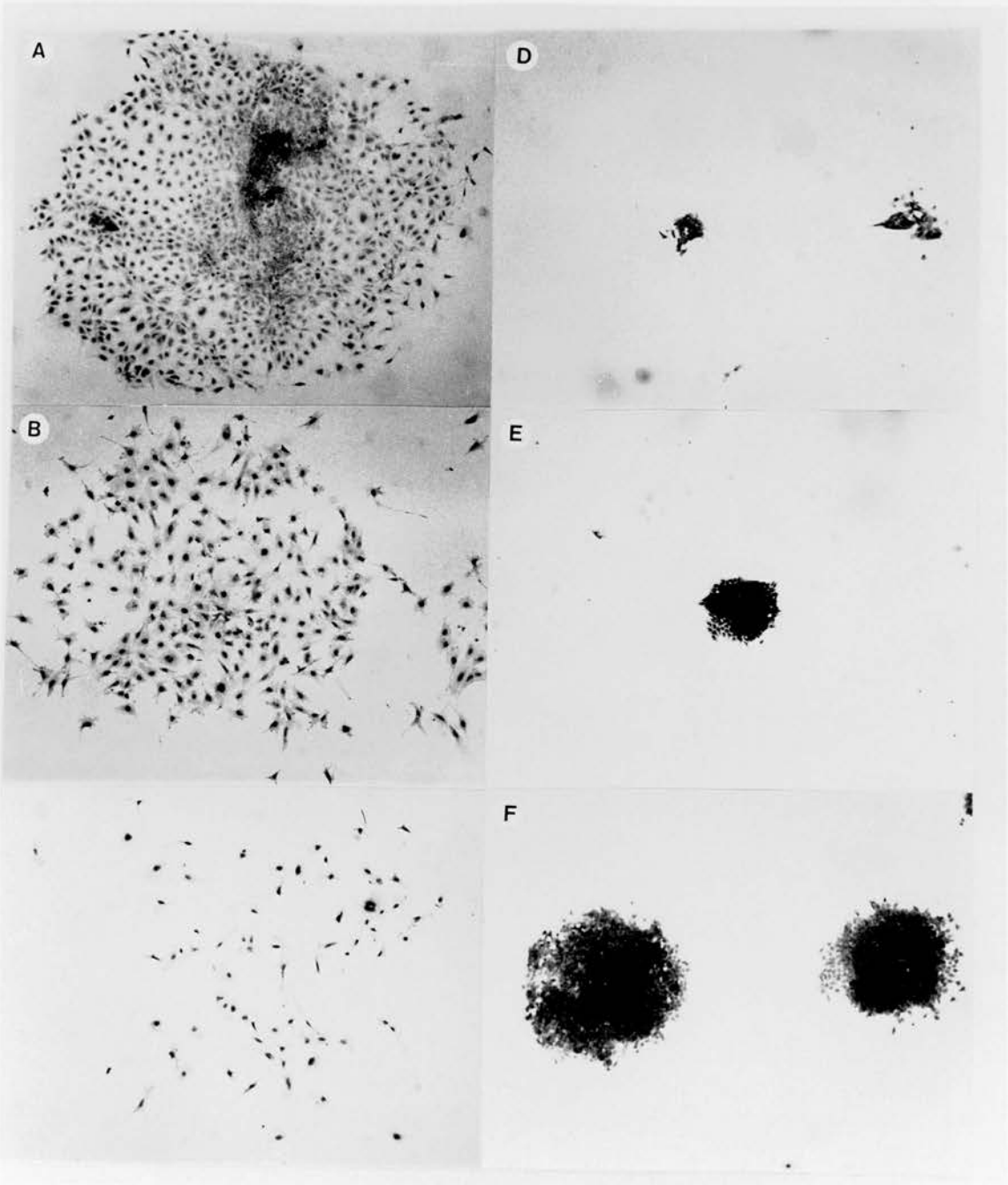
PT2md1 is not unaffected by chemical inducers of differentiation, however. Both retinoic acid and HMBA cause very high levels of cell death. The mortality of PT2md1 appears to mirror differentiation of PT2. Conditioned media which inhibit the induction of differentiation of PT2 reduce the toxicity of retinoic acid and HMBA towards PT2md1. It would be of interest to compare the duration of exposure to retinoic acid required for induction of the two effects. Involvement of the mec^- phenotype in the high mortality of PT2md1 seems unlikely given that the EC line R5/3 (Slack *et al*, 1978), which shows a greater reduction in junctional communication than PT2md1, is not killed by retinoic acid but differentiates normally. This possibility might be formally excluded by investigating the response to retinoic acid of a recently isolated revertant of PT2md1 which shows normal metabolic cooperation properties but remains incapable of differentiation (M.L.Hooper, personal communication).

An alternative explanation for the different mortalities of the two cell lines in retinoic acid might be that PT2 is unusually resistant and PT2md1 displays the normal susceptibility. The occurrence of such a spontaneous variation followed by a reversion without selection is highly improbable. It is mentioned because the toxic effects of retinoic acid towards PT2, as depicted in Fig.4.2 (mean $L.D._{50} > 10^{-5}M$), are markedly different from its actions on PSA4TG12 reported in Section 3.2.2. (mean $L.D._{50}=1.1x10^{-9}M$). However, different sera were used in these two sets of experiments, newborn calf serum in the former and foetal

Plate 4.4 PT2 and PT2mdl Colonies in Various Media

Plating tests were set up as described in Section 2.9. After 6 days cultures were fixed and stained with Leishman's:-

	Magnification
A. PT2 in CM containing 10% foetal calf serum	30
B. PT2 in CM containing 10% newborn calf serum plus 3mM HMBA	30
C. PT2 in CM containing 10% newborn calf serum	30
D. PT2mdl in CM containing 10% newborn calf serum	30
E. PT2mdl in BRL-medium containing 10% newborn calf serum	30
F. PT2 in BRL-medium containing 10% newborn calf serum	30


A
D
B
E
F

calf serum in the latter. When toxicity of retinoic acid towards PT2 was determined in medium containing 10% foetal calf serum it showed a similar concentration-dependent decrease in cloning efficiency as PSA4TG12 and a comparable $L.D._{50}$ ($1.6x10^{-9}M$). PT2 thus exhibits a similar phenotype to PSA4TG12 in this respect as in others (Smith, 1984) and it is PT2md1 which shows a variant character. It should be noted in passing that the spontaneous differentiation of PT2 and other PSA4-derived EC cells in the absence of feeders appears different, in terms of the number and form of differentiated cells obtained, in foetal calf serum-containing medium compared with medium containing newborn calf serum. The morphology of the majority of differentiated cells obtained in the two media is quite distinct; large colonies of flattened, closely apposed cells predominate in foetal serum (Plate 4.4A), whereas small clusters of fibroblastic cells displaying varying degrees of cell-cell contact are the major product in newborn serum (Plate 4.4C). Interestingly, HMBA induces differentiation in newborn calf serum more like the form normally found in foetal calf serum. Retinoic acid, in contrast, does not have any conspicuous effects on the spectrum of differentiated morphologies obtained in newborn serum while its effects on differentiation in foetal serum are unclear due to its high toxicity (Section 3.4.3.). It would be of considerable interest to determine whether the different morphologies represent distinct cell types or are alternative configurations of a single cell type.

The preceding observations underline the important though ill-understood effects of serum factors on embryonal carcinoma proliferation and differentiation (see Section 1.7.). The differences in toxicity are too great to be accounted for simply by differences in endogenous levels of retinoids or retinoid binding proteins in the two sera. They may be related to the apparently different effects of foetal and calf serum on spontaneous differentiation. For example, following induction with retinoic acid foetal calf serum may promote a differentiation pathway culminating in cell death (see below). Alternatively, retinoic acid may be highly toxic to the type of differentiated cell obtained in foetal serum whereas this is clearly not the case for differentiated cells in newborn serum. Various other explanations can be conceived involving interactions of retinoic acid with various serum factors, but these would be difficult to test given the complex and poorly characterised nature of whole serum.

The evidence presented above favours the hypothesis that the mortality of

PT2md1 in the presence of chemical inducers results from a deficient differentiation response. The fact that other differentiation-deficient EC lines which do not respond to inducing agents (Schindler *et al*, 1981; Jones-Villeneuve *et al*, 1983; McCue *et al*, 1983; Wang and Gudas, 1984; Moore *et al*, 1985) have not been reported to show any heightened susceptibility towards the toxic effects of these chemicals may appear to argue against this idea. However, all these lines were isolated by procedures similar to that employed in Section 4.3.1. involving specific selection for the ability to proliferate in the presence of the inducer. *Ipso facto*, all clones obtained in this way are resistant to any toxic effects of the chemicals. PT2md1, in contrast, was isolated without exposure to inducing agents and this presumably underlies its unique phenotype.

There are at least three ways in which the inability to differentiate may result in high mortality in the presence of chemical inducers:-

1. The inducers may be directly toxic to undifferentiated stem cells.
2. Cell death may represent abortive differentiation.
3. Cell death may be a normal form of differentiation favoured by these cells.

In the first explanation mortality is quite independent of the differentiation process. This is argued against by the finding that conditioned medium increases the proportion of EC cells in PT2 cultures but reduces the toxic effects of retinoic acid and HMBA; in BRL-medium PT2 grows as a near-homogeneous population of embryonal carcinoma, yet colony formation in the presence of retinoic acid or HMBA is increased. These observations cannot be explained by inactivation of the inducers as retinoic acid remains active in feeder- and BRL-conditioned medium (Smith and Hooper, 1983; Pitts *et al*, in press). Indeed it induces some differentiation of PSA4TG12 and PT2 in conditioned medium at concentrations as low as 10^{-9}M (Fig. 3.1 and Table 4.8). It is conceivable that both types of conditioned medium could have two separate actions; inhibition of differentiation, and protection against the toxic effects of two unrelated chemicals. Preliminary evidence against this hypothesis is provided by the observation that retinol, which is expected to be similar to retinoic acid in its cytotoxic actions (Lotan, 1980), does not appear to

affect the mortality of PT2 and PT2md1 differentially except under conditions in which it promotes differentiation of the former. It is necessary to confirm this finding, however, by carrying out a full dose-response analysis of the effects of retinol in normal and conditioned medium. It would also be valuable to examine the effects of higher concentrations of HMBA in BRL-medium. In both cases, if selective mortality of PT2md1 is dependent on cell commitment, the mortality of the two cell lines should increase in parallel in the absence of differentiation. Conversely, if and when differentiation is induced, the mortality of PT2md1 should exceed that of PT2.

The second and third explanations both assume an association of cell mortality with the commitment to differentiate. The only contraindication is the survival of PT2md1 in conditions which lead to the spontaneous differentiation of PT2. As described in Section 4.5.2., PT2md1 proliferates in the absence of feeders in normal medium containing 2-mercaptoethanol. This could be because it is unresponsive to the stimuli which induce a so-called "spontaneous" commitment to differentiate. However, feeder-free culture of PT2md1 does entail higher levels of cell death and growth to confluence takes longer than for true feeder-independent EC lines like PC13.5. BRL-medium enhances the survival of PT2md1 in the absence of feeders such that its growth appears comparable to that of PC13.5. In plating tests PT2md1 colonies generally contain fewer cells after 1 week than PT2 colonies, despite the occurrence of differentiation in the latter (Plate 4.4). Conditioned media enhance colony formation by both cell lines. They also increase the mean colony size (Plate 4.4), which reflects cell growth rate (see Section 7.3.). These actions of conditioned media might be due to generalised effects on EC cell viability, mediated via inactivation of toxic substances or the presence of a growth factor, for example. Equally, however, they could be directly related to the inhibition of spontaneous differentiation, which promotes EC cell proliferation in PT2 cultures and conversely might reduce EC cell death in PT2md1 cultures

The poorer growth of PT2md1 in medium containing foetal calf serum rather than newborn calf serum is contrary to what is generally observed for EC cells. In view of the apparent effects of serum on differentiation mentioned above, this may lend support to the suggestion that as PT2 undergoes spontaneous differentiation, PT2md1 undergoes some level of cell death. If this is the case, however, there does not seem to be a simple one-to-one relationship between the two phenomena as colony generation by the two lines is similar in normal

medium containing newborn serum, yet only one third of PT2 colonies retain undifferentiated cells (though some of the PT2 cells probably become committed to differentiate during the previous passage in feeder-conditioned medium).

Whether or not spontaneous commitment occurs and leads to any cell death of PT2md1, it is obvious that exposure to chemical agents results in far higher mortality. This is probably because chemicals such as retinoic acid and HMBA are more potent inducers of commitment than so-called "spontaneous" stimuli. Thus retinoic acid causes differentiation of EC lines such as PC13.5, which show little or no spontaneous differentiation *in vitro*, and Nulli-1, which does not even differentiate *in vivo*.

As pointed out earlier, the induction of cell death could be due to a defective differentiation event or may be an alternative form of differentiation which is accentuated in PT2md1 because other pathways are blocked. It is difficult to envisage any direct method of discriminating between these two hypotheses as the distinction between them is only significant in the context of normal embryogenesis.

Certainly cell death is widespread during embryogenesis and its occurrence in many cases is clearly predetermined (Saunders, 1966). The embryonic counterparts of EC cells are the pluripotent epiblast cells of the implantation stage embryo (Section 1.3.). There is considerable cell death in this lineage, both in the inner cell mass (ICM) prior to implantation (El-Shershaby and Hinchliffe, 1974) and in the primary ectoderm after implantation (Poelmann and Vermeij-Keers, 1976; Poelmann, 1980). It has been proposed that cell death in the ICM is a homeostatic mechanism for regulation of cell number and synchronization of cell division (Handyside and Hunter, 1984). Regulation of cell numbers may also be a role of cell death in the primary ectoderm. In the latter case, however, it may have a further function in tissue-modelling (Bonnevie, 1950; Martin *et al* 1977). Evidence for this comes largely from observations on the embryoid body structures formed by certain EC lines when aggregated in suspension culture (Section 1.4.). In some cases these embryoid bodies undergo eccentric cavitation and it has been proposed that this parallels formation of the proamniotic cavity in the post-implantation embryo. Martin *et al* (1977) described the incidence of reproducible and focal cell death associated with cavity formation in embryoid bodies.

Cells may die by two morphologically distinct pathways; necrosis and apoptosis (Wyllie, 1981). Boyd *et al* (1984) have presented evidence that both types of cell death occur in cavitating embryoid bodies but that it is necrosis which is specifically associated with cavity formation. The prospective cavity in an embryoid body first becomes recognizable as an area of dying cells (Martin *et al*, 1977). This suggests that necrosis may be the primary event in cavity formation, though the possibility cannot be excluded that necrosis and cavitation are parallel responses to a common stimulus. It seems clear from these studies on embryoid bodies that EC mortality can be developmentally regulated and this is reflected in the adoption of a particular mode of cell death. It would therefore be of interest to determine whether PT2md1 cells become necrotic or apoptotic in response to retinoic acid. Aggregates formed from PT2md1 do not develop into embryoid bodies. Nor do they undergo cavitation (Smith, 1984), presumably because they lack the outer layer of endoderm which is believed to control this process (Martin *et al*, 1977). The effects of retinoic acid and HMBA on aggregates of PT2md1 have not been investigated and such a study might prove worthwhile. It is conceivable that the chemicals might induce focal cell death and cavity formation. It should be noted that further differentiation of the EC core of embryoid bodies only occurs in the presence of cavitation, indicating that there may be an intimate association between the incidence of cell death and cell commitment.

That the phenomenon of EC mortality may be genetically determined is evidenced by the results of a time-lapse film analysis of retinoic acid-induced differentiation of PC13 EC cells (Mummery *et al*, 1984). These workers found that early events in the differentiation process were accompanied by a significant level of cell death. Cells frequently lysed as sister pairs suggesting that mortality was preprogrammed. The incidence of cell death was low during the first two cell cycles after addition of retinoic acid but rose dramatically during the third cycle, coinciding with an increase in generation time but succeeding initial morphological changes associated with differentiation. This high mortality level is not a general characteristic of differentiated EC-derivatives but appears to be specifically associated with the induction of differentiation. The finding that for sister pairs filmed during the third cycle after retinoic acid addition, both survived until the next generation in 49% of cases while both cells lysed, and furthermore did so within 20 minutes of one another, in 30% of cases, indicates that cells probably become committed to

die in the generation prior to lysis. This is supported by the observation that it is possible to predict during the second cell cycle those cells which will eventually give rise to the differentiated population and those whose descendants will die; cells which acquire a more flattened morphology characteristic of the differentiated phenotype generally survive, while cells which flatten less extensively often die during the following cell cycle. Unfortunately these workers do not report whether the cell death they observed proceeded via apoptosis or necrosis.

There is evidence therefore that cell death may be a normal programmed fate of embryonal carcinoma cells rather than merely an aberration resulting from an unfavourable environment or defective genetic disposition. PT2 may prove to be a useful tool for investigating this issue further. A time-lapse film analysis of the effects of retinoic acid and HMBA would be invaluable in determining the temporal relationship between death of PT2md1 and differentiation of PT2 and confirming whether the elevated mortality of the former proceeds via a commitment to die. If the fate of PT2md1 is genetically determined, it would provide an *in vitro* system for analysing developmentally programmed cell death. Studies of gene and protein expression could then be employed to elucidate the mechanism of this process in the same way as such techniques are used to probe 'conventional' differentiation into different cell types. Alternatively, if PT2md1 exhibits a lethal defect in the differentiation program, it may be possible to determine the site of this lesion and thus identify an essential component of the machinery of cellular differentiation.

4.6. GENERAL DISCUSSION

Various aspects of the interaction of retinoic acid with embryonal carcinoma cells have been examined in this Chapter. The main aim has been to shed light on the mechanism by which retinoic acid induces differentiation of these cells. Limited progress has been made towards this objective and avenues for further research are suggested.

The ouabain rescue data for PC13.5 in the presence of retinoic acid (Section 4.2) indicate that differentiation is not induced via inhibition of metabolic cooperation and when taken in conjunction with the observations on PTmr0 differentiation in the previous Chapter the evidence becomes compelling. They

further indicate that the induction of differentiation and the inhibition of metabolic cooperation occur via separate pathways (hypothesis 3 in Section 3.4.). This is suggested by the massive difference in concentration required for the two effects and would seem to be confirmed by the finding that conditions causing significant inhibition of the former, i.e. culture on a feeder layer, are apparently without effect on the latter. Thirdly, these results argue against any involvement of CRABP in the inhibition of junctional communication since the latter only becomes manifest at concentrations three orders of magnitude greater than the K_d for the RA-CRABP complex. This possibility can only be formally excluded, however, by experiments on a variant EC line lacking functional CRABP.

Following continuous culture in 10^{-5}M retinoic acid, two EC clones have been isolated which show a significantly reduced differentiation response. The first of these, PRA0.1, was derived from a non-mutagenized PC13.5 stock. It does undergo some morphological and biochemical changes on exposure to retinoic acid but these do not culminate in terminal differentiation as occurs with the parental line. Subcloning experiments are necessary to determine whether the partial differentiation observed is a result of heterogeneity within the population or whether, as is suggested by the apparent uniformity of the response, it represents an incomplete reversible differentiation. A variant of the latter type has recently been described by Moore *et al* (1985). This was isolated from F9 EC cells by a selection procedure very similar to that employed here. Such variants are potentially of value in dissecting the various steps of the pathway by which differentiation proceeds. In this regard it may already be noted that the response of PRA0.1 to retinoic acid appears to differ in some respects from that described for the variant isolated by Moore *et al* (1985). The nature of the limited differentiation undergone by PRA0.1 could, therefore, be of interest in its own right. In particular, the apparent occurence of dedifferentiation is worthy of further investigation and if confirmed should be characterised extensively in order to identify those differentiation events which are reversible.

The second differentiation-deficient clone described in Section 4.3. was derived from mutagenized PC13.5 stock. PRA7c differs from PRA0.1 in that it is essentially non-responsive to retinoic acid. It is thus similar to the variants obtained by Schindler *et al* (1981) and McCue *et al* (1983). Presumably the lesion in such cell lines is at the stage of interaction with retinoic acid and

initiation of commitment. They are potentially of value in elucidating the mechanism by which cells become committed and the mode of action of retinoic acid (Schindler *et al*, 1981; McCue *et al*, 1983; Sherman *et al*, 1983; McCue *et al*, 1984a,b). Some variants of this type lack, or have greatly reduced, levels of CRABP (Schindler *et al*, 1981).

An important question in developmental biology is whether there is a unique mechanism for inducing differentiation of embryonic stem cells or whether multiple inductive systems coexist. With regard to epiblast cells, it might be argued that the response of EC cells to retinoic acid is purely artefactual and bears no relation to processes of commitment and differentiation operating in the embryo (but see following Chapter, Section 5.3.6.). At the other extreme of this proposition would be the assertion that retinoic acid is the embryological inductive agent which controls differentiation of the ICM and primary ectoderm *in vivo* precisely as it does *in vitro* (see Section 1.5. for further discussion). Differentiation-deficient variants can be employed to investigate whether alternative mechanisms of EC cell commitment exist *in vitro* or *in vivo*. Thus McCue *et al* (1983) have presented evidence that HMBA induces differentiation via some unidentified interaction with the retinoic acid pathway, whilst McBurney and co-workers (McBurney *et al*, 1983; Edwards *et al*, 1983; Jones-Villeneuve *et al*, 1983; Campione-Piccardo *et al*, 1985a,b) report that the induction of commitment of P19 EC cells by retinoic acid or DMSO proceeds via distinct mechanisms. A variety of chemicals have been reported to induce differentiation of EC cells (Section 1.5.). Several of these inducing agents, namely DMSO, HMBA, butyrate and polybrene, were tested on PC13.5 cells. Unfortunately none of them caused overt differentiation (though HMBA and polybrene caused minor reversible changes in morphology). Consequently PRA0.1 and PRA7c are probably of little use for the type of study referred to above. Polybrene does appear to enhance the action of retinoic acid on differentiation of PC13.5 (Heath and Deller, 1983) but the effects of this combination on PRA0.1 and PRA7c are unclear because, although some differentiated cells appear, there is a very high level of cell death, particularly of PRA7c. This might be worthy of further investigation in view of the proposition of a connection between differentiation and mortality (Section 4.5.3.).

Tumour analyses suggest that differentiation of PRA0.1 may be restricted *in vivo* as *in vitro*, indicative perhaps of a fundamental lesion during the

differentiation process. The developmental potential of PRA7c *in vivo* remains unknown as it is apparently not tumourigenic in immunocompetent hosts. It is possible that PRA7c might give rise to tumours in immunodeficent mice (Boon *et al*, 1974), however, and if so this would provide a means of determining whether an alternative mechanism of commitment is operational *in vivo*.

It has been postulated that possession of CRABP underlies the responsiveness of EC cells to retinoic acid (Jetten and Jetten, 1979; Sherman *et al*, 1981). Consistent with this are reports that, with the exception of the aforementioned variants isolated by Schindler *et al* (1981) and one of the clones selected by McCue *et al* (1983), all EC cells tested possess CRABP. This finding is here extended to feeder-independent PC13 derivatives and feeder-dependent PSA4-derived EC lines which are routinely cultured in this and other laboratories. If retinoic acid-induced differentiation is mediated via CRABP, it follows from the preceeding discussion on the nature of the lesion in the two cell lines that PRA0.1 should exhibit this activity whilst PRA7c might not. In fact both clones display saturable binding of retinoic acid. Therefore the lesion in PRA7c is not at the level of CRABP expression. CRABP is also manifest in two variants with altered junctional communication properties, PTmr0 (see previous Chapter) and PT2md1. The latter finding is of interest since it is consistent with a potential involvement of the binding protein in the induction of cell death by retinoic acid (Section 4.5.3).

The characterization of PT2md1 in Section 4.5. reveals that this clone has a very unusual phenotype. The simplest explanation for the failure of PT2md1 to differentiate spontaneously, in aggregates or in monolayers, or to undergo chemically induced differentiation is that due to some mutation(s) it has completely lost the capacity for differentiation. It would be of interest to examine tumours formed from this cell line to see whether it displays any developmental potential *in vivo*.

An account has been given here and by Smith (1984) of how the procedure employed to isolate PT2md1 may select for a non-differentiating (dif^-) phenotype. It is implicit in such explanations that the mec^- and dif^- characters of PT2md1 are independent. The finding that PT2md1 does not differentiate in retinoic acid provides strong support for this since there is no evidence to suggest that retinoic acid-induced differentiation might be dependent on metabolic cooperation. Indeed the mec^- clone R5/3 (Slack *et al*, 1978), which

shows a greater reduction in junctional communication capacity than PT2md1, undergoes extensive differentiation on exposure to retinoic acid (Hooper, 1982b). The distinct nature of the dif$^-$ lesion in PT2md1 appears to be confirmed by the isolation of a revertant with restored cooperation properties which does not differentiate in embryoid bodies (M.L. Hooper, personal communication). Such a conclusion still permits the possibility of a role for junctional communication in some modes of EC cell differentiation, however (Hooper, 1982b).

The response of PT2md1 to retinoic acid and HMBA appears to be unique. Retinoids and other chemical inducing agents often cause some level of cell death but only one other EC line, PCC4aza1, has been reported to show an acute susceptibility to retinoic acid toxicity (Strickland and Mahdavi, 1978; Jetten *et al*, 1979; Sherman *et al*, 1983). In other respects the phenotype of PCC4aza1 is similar to that of PSA4TG12 and PT2. Thus it undergoes spontaneous differentiation in monolayers and in aggregates (Lo and Gilula, 1980a,b) and this differentiation is promoted by several chemicals including HMBA (Speers *et al*, 1979). PSA4TG12 and PT2 are highly susceptible to retinoic acid in CM containing foetal rather than newborn serum for reasons which are not clear (see discussion in Section 4.5.4.). The mortality of PCC4aza1 may likewise depend on factors present or lacking in the medium. At concentrations of retinoic acid below 10^{-8}M PCC4aza1 shows morphological evidence of differentiation (Strickland and Mahdavi, 1978). Its capacity for mounting a differentiation response is confirmed by the isolation of a subclone, PCC4aza1R, which survives and differentiates in retinoic acid concentrations from 10^{-9}–10^{-5}M (Jetten *et al*, 1979, Sherman *et al*, 1981).

PT2md1, in contrast, seems to be incapable of differentiation and it has been argued that this underlies its fatal response to two chemically distinct inducers of differentiation, retinoic acid and HMBA. This case would be strengthened if further promoters of PT2 differentiation were identified and shown to be toxic to PT2md1. Unfortunately, neither polybrene nor sodium butyrate appear to have any effect on differentiation, though the latter does slow cell growth. Another approach would be to find a more precise and quantitative index of differentiation of PT2, which could be employed in normal and conditioned media. This could be used to substantiate or otherwise the apparent correlation between induced differentiation of PT2 and mortality of PT2md1. It is difficult to conceive of any direct measure of the effect of retinoic acid in

unmodified medium since it does not seem to alter the spectrum of spontaneous differentiation. A possible indirect assay would be to examine loss of cloning efficiency after exposure to retinoic acid (Campione-Piccardo *et al*, 1985a). HMBA, in contrast, seems to induce the appearance of a particular cell form (Plate 4.4B), so it may be possible to quantify directly the specific differentiation response induced by this agent. It might also prove informative to compare the duration of exposure to the inducers required to elicit a differentiation response in PT2 and to induce cell death in PT2md1.

For the present, however, the available evidence indicates that factors which induce or inhibit the induction of differentiation of normal EC cells, likewise induce or inhibit the induction of PT2md1 cell death. These observations are consistent with an association between commitment and cell death. In line with this is the finding, reported in the previous Chapter, that PTmr0 apparently has a decreased sensitivity to both the differentiation-inducing and toxic effects of retinoic acid. This idea could be confirmed by time lapse film analyses of the temporal relationship between commitment and cell death. Such studies could also be used to investigate whether death of PT2md1 arises from a lethal defect in the mechanism of commitment or differentiation, or whether it corresponds to a normal differentiation pathway which might be of condiderable importance in early embryonic development.

CHAPTER 5

THE EFFECTS OF MEDIUM CONDITIONED BY BUFFALO RAT LIVER CELLS ON MURINE EMBRYONAL CARCINOMA CELLS

5.1. INTRODUCTION

Both spontaneous and retinoic acid-induced differentiation of EC cells are greatly reduced by culture on feeder layers of growth-arrested embryonic fibroblasts (Martin and Evans, 1975b; Ogiso *et al*, 1982). This effect is mediated at least partly via contact-independent mechanisms, since medium conditioned by preincubation with an STO feeder layer inhibits differentiation (Smith and Hooper, 1983). Such activity is present in medium conditioned by a variety of cell types and is not dependent on mitotic arrest (Koopman and Cotton, 1984). However, evidence has also been presented for the importance of cell-cell contacts in the interaction between EC and feeder cells (Isacke and Deller, 1983). Indeed, conditioned medium is only partially effective in inhibiting the differentiation of feeder-dependent EC cells plated on gelatinized substrata. Thus, although it is possible to maintain stocks of feeder-dependent EC lines in the absence of feeder layers using conditioned medium, such cultures are heterogeneous and generally contain a significant proportion of differentiated cells of various types (Koopman and Cotton, 1984; see Section 4.5.2. and Section 5.2.). Moreover, reproducibility between batches of feeder-conditioned medium is variable, presumably reflecting differences between feeder preparations. A more potent and consistent "differentiation inhibiting activity", which enabled feeder-free clonal growth and maintenance of homogeneous stem cell populations of otherwise feeder-dependent EC lines, would demonstrate that cell contacts do not play an essential role in the phenomenon of feeder-dependence. It would also be of great practical benefit in the culture of EC and EK cells. In this Chapter the identification and characterization of such an activity are described and some of the implications of this discovery are discussed. Partial purification of the activity is reported in the following Chapter.

5.2. INITIAL OBSERVATIONS

Various factors have been reported to promote the survival and proliferation of teratocarcinoma and pluripotent embryonic stem cells *in vitro*, including 2-mercaptoethanol (Oshima, 1978), detoxifying agents (J.Heath, personal communication), insulin and insulin-like growth factors (Rizzino and Crowley, 1980; Nagarajan *et al*, 1982) and factors produced by EC cells themselves (Martin, 1981; Heath *et al*, 1984; Heath and Isacke, 1984).

Media containing the above activities were screened for their effects on growth and differentiation of the feeder-dependent EC line PT2 (Smith, 1984) in the absence of feeders. These experiments were performed in CM containing 10% foetal calf serum. In all cases 2-mercaptoethanol is present as this is essential for viability of monolayer cultures of PSA4-derived EC lines on gelatinized substrata (see Chapter 7). The detoxifying agents employed are catalase and mannitol which prevent accumulation of free radicals in the medium. Medium conditioned by PC13.5 EC cells (Hooper and Slack, 1977) should contain embryonal carcinoma-derived growth factor (ECDGF) (Heath and Isacke, 1984). This factor has been reported to inhibit the spontaneous differentiation of OC15S2 EC cells (Heath *et al*, 1984). In addition, EC cell-conditioned medium has been reported to promote the growth of embryonic stem cells *in vitro* and has been employed in the isolation of EK lines (Martin, 1981; Martin and Lock, 1983). Buffalo rat liver (BRL) cells (Coon, 1968) produce large amounts of insulin-like growth factor-II (IGF-II) (Marquardt *et al*, 1981), previously known as multiplication-stimulating activity (MSA) (Dulak and Temin, 1973a). Physiological concentrations of this growth factor support proliferation of F9 and PC13 EC cells in serum-free medium (Nagarajan *et al*, 1982; Heath and Deller, 1983). Data from plating tests (Section 2.9.) carried out in the different media are presented in Table 5.1.

Table 5.1
Colony Formation by and Differentiation of PT2 in Various Media

Medium	Relative Number of Colonies (%)	Differentiation (%) EC	Mixed	Diff^
A	100.0	0	78	22
B	98.2	1	72	27
C	162.4	8	76	16
D	62.8	0	28	72
E	133.5*	99	0	1

^Differentiated
*Colonies small in size,<50 cells on average

Dishes, fixed and stained with Leishmans' after 6 days, were scored both for total colony number and for the differentiation status of the individual colonies as assessed by light microscopy.
Data are the means of determinations on triplicate dishes. Media are:-

A. CM
B. CM + 5µg/ml catalase + 10nM mannitol
C. STO feeder-conditioned CM
D. PC13.5-conditioned CM
E. BRL-conditioned CM

Conditioned media were prepared by incubating 30ml of CM per 175cm^2 flask of mitomycin C-treated STO cells or exponentially growing PC13.5 or BRL cells for 3 days. The media were sterile-filtered and used with no additions.

The mean number of colonies formed per 1000 cells seeded in unmodified CM was 295 in these experiments. The proportion of colonies retaining EC cells in CM is greater than reported in the previous Chapter (Section 4.5.3.), probably as a conseqence of the use of foetal rather than newborn calf serum (see discussion in Section 4.5.4.).

Feeder-conditioned medium enhances colony formation by PT2 and restricts differentiation in that the majority of colonies are composed predominantly of cells of EC morphology. Nonetheless, over 90% of colonies contain some overtly differentiated cells.

One activity which has been proposed for feeder cells is the detoxification of harmful constituents in culture medium (Isacke and Deller, 1983). However, neither colony generation nor differentiation are significantly affected by the presence of catalase and mannitol. Protection against free radicals is therefore unlikely to be a major function of feeder layers, though this does not preclude some other detoxifying role.

Colony formation by PT2 is reduced in medium conditioned by PC13.5 EC cells and differentiation appears to be promoted. The former effect may be a consequence of depletion of nutrients from, or accumulation of toxic metabolites in, the medium. The reduced proportion of residual EC cells may be related to deterioration of the medium leading to EC cell death and/or stimulation of differentiation. An apparent enhancement of differentiation could also reflect the growth-promoting activity of ECDGF on differentiated teratocarcinoma derivatives (Heath and Isacke, 1984).

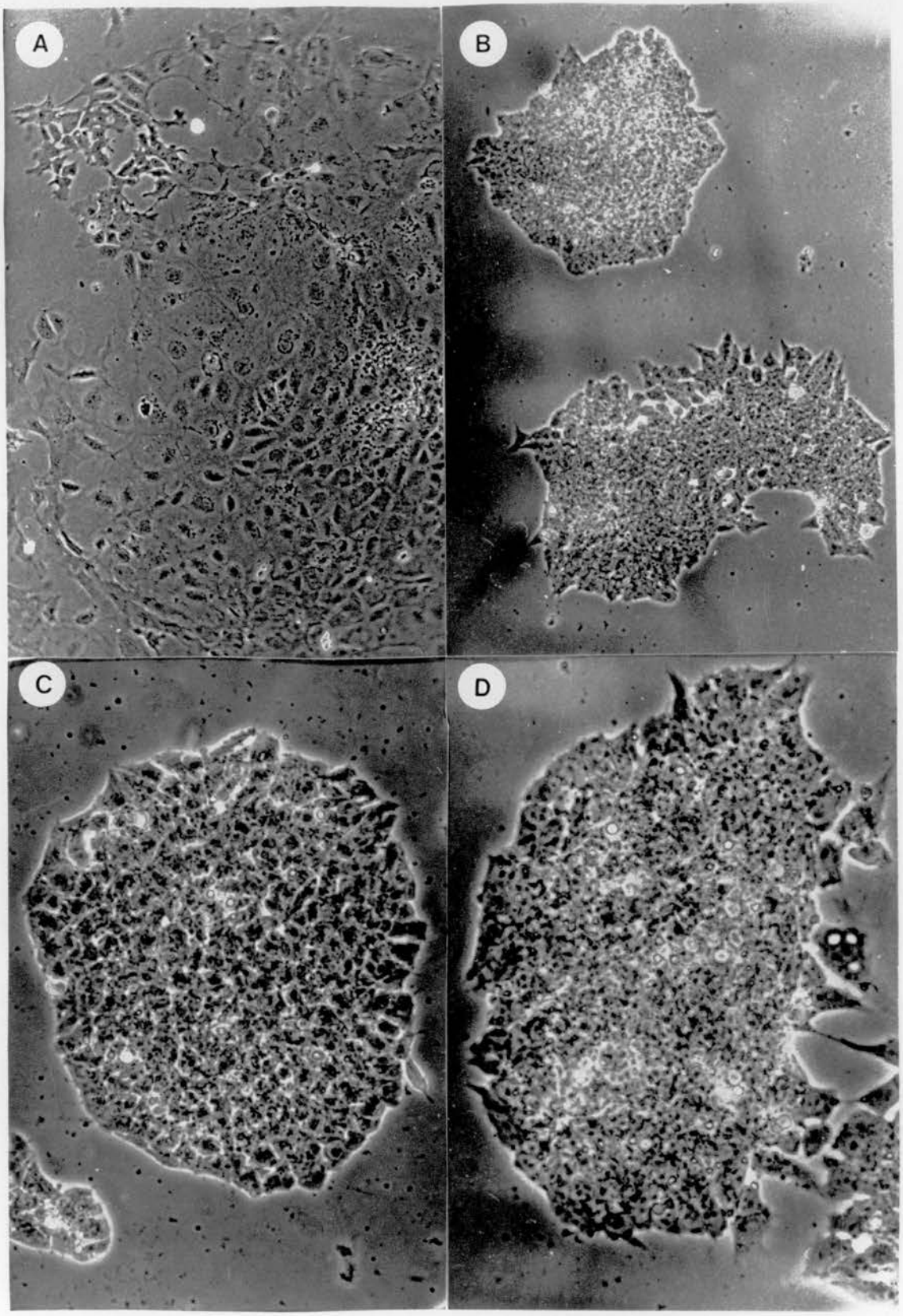
In sharp contrast to the above, BRL-conditioned medium both improves plating efficiency and dramatically reduces differentiation. Whereas in other media almost all colonies consist wholly or partly of large, flattened cells and those EC cells which remain are quite spread and have prominent nuclei, in BRL-conditioned medium the colonies consist of small, tightly packed, intensely staining cells with indistinct nuclei, resembling the appearance of PC13.5 colonies under optimal conditions (Plate 5.1; see also Plate 4.4F). Less than 1% of the cells display any morphological indication of differentiation. The colonies do contain fewer cells than in other media, however.

The conclusions drawn from plating tests are corroborated by observations on mass cultures of PT2 established in $25cm^2$ flasks as detailed in Section 2.10.2. After 6 days, cultures in CM and in CM plus catalase and mannitol have attained confluence and more than 90% of the cells are overtly differentiated (Plate 5.1A). Cells in PC13.5-conditioned medium show a similar if not greater degree of differentiation, though cell number is lower. In feeder-conditioned medium monolayers are nearing confluence and consist predominantly (>80%) of EC cells. A significant subpopulation of differentiated cells is clearly present, however. Flasks maintained with BRL-conditioned medium contain scattered small colonies, over 90% of which appear to be pure embryonal carcinoma (Plate 5.1B). Differences in degree of confluence are partly attributable to differences in cell size, since differentiated cells occupy considerably more surface area than EC cells. Notwithstanding this, there appears to be a reduction in cell number in BRL-medium relative to the other media. This is not due to any acute toxic effect since plating tests established that colony formation is increased compared with normal medium (Table 5.1). Moreover, surviving cells appear quite healthy and there is no evidence of extensive cell death. Indeed mortality seems lower in BRL-conditioned medium than in differentiating cultures, if judged by the numbers of detached cells floating in

Plate 5.1 Feeder-Free Culture of PT2 in BRL-Conditioned Medium

Phase contrast

	Magnification
A. PT2 in CM, 6 days	134
B. PT2 in 100% BRL-conditioned medium, 14 days	168
C. PC13.5 in CM	430
D. PT2 in 100% BRL-conditioned medium, 14 days	484


A
B
C
D

the medium.

The absence of any significant increase in mortality argues against the effects of BRL-medium on EC cell proliferation and differentiation being mediated via selective killing of differentiated cells (see also Section 5.3.3. below). However, if commitment only occurs in cycling cells, the lack of differentiation could be an artefact arising from the arrested growth of the cells. Consequently, BRL-conditioned media were prepared in various ways in an attempt to find conditions which would permit EC cell growth and prevent differentiation. The results of screening these different media on mass cultures are presented in Table 5.2.

EC cell proliferation could be blocked either by depletion of essential constituents from the medium or via the action of a growth inhibitory activity released by the BRL cells (see Section 6.3.). Either or both of these factors might be dependent on growth of the BRL cells. Likewise a specific "differentiation inhibiting activity" might only be produced by actively dividing cells. Multiplication of BRL cells is strongly inhibited at high cell density and they become quiescent at confluence. However, PT2 cultures respond similarly to medium conditioned by confluent BRL monolayers as to medium conditioned by exponential phase cultures. Moreover, it is possible to repeatedly harvest these stationary cultures of active conditioned medium for at least 4 weeks. Furthermore, the activty is stable to freezing and thawing and can be stored at -20°C for up to three months.

Increasing up to three-fold the volume of medium conditioned by a flask of BRL cells only slightly improves cell growth. However, when BRL-conditioned medium is diluted with normal medium PT2 cultures proliferate progressively. At concentrations of BRL-conditioned medium between 50% and 75% (v/v) differentiation is negligible and growth rate comparable to that of truly feeder-independent EC lines such as PC13.5, that is cultures seeded at a density of $4x10^3$ on Day 1 are confluent by Day 7, representing a greater than 100-fold increase in cell number.

Table 5.2
The Effects of Various Preparations of BRL-Conditioned Medium on Growth and Differentiation of PSA4 and PSA4 Derivatives in Monolayer Culture

Conditions of Preparation*	Final Concentration^	Growth Rate	Inhibition of Differentiation
Confluent BRL culture[a]	100	Arrested	+++
" "	75	Intermediate	+++
" "	70	Rapid	+++
" "	60	Rapid	+++
" "	50	Rapid	++
4 week confluent culture[b]	100	Arrested	+++
" "	60	Rapid	+++
Storage at -20°C[c]	100	Arrested	+++
" "	60	Rapid	+++
Sub-confluent BRL culture	100	Arrested	+++
" "	50	Rapid	++
50ml/flask	100	Poor	+++
90ml/flask	100	Poor	++
Newborn calf serum	100	Poor	+++
" "	75	Rapid	++
" "	50	Rapid	+
Serum-free,plus FCS[d]	100	Arrested	+++
" "	75	Poor	+++
" "	50	Rapid	++
Serum-free,plus NCS[e]	75	Rapid	+
Plus FCS and Gln[f]	100	Arrested	+++

*BRL-conditioned medium was prepared from CM containing foetal calf serum (except where otherwise specified) as described in Section 2.7. with the indicated modifications
^% (v/v) BRL-conditioned medium in final culture medium (diluted with CM)
[a]Equivalent to standard conditions specified in Section 2.7.
[b]4 week old culture previously harvested several times
[c]Standard preparation frozen and stored for up to 3 months
[d]Foetal calf serum (10% v/v) added after conditioning
[e]Newborn calf serum (10% v/v) added after conditioning
[f]Additional foetal calf serum (10% v/v) and glutamine (2mM) added after conditioning

Cultures,previously passaged at least once in the absence of feeders, were seeded at a density of $4x10^3/cm^2$ on gelatinized substrata in the medium under test (Day 1). They were refed with the same medium on Day 4. Differentiation was assessed by morphological criteria on Day 7:-

+++ less than 5% of cells differentiated
++ 5-10% of cells differentiated
+ 10-20% of cells differentiated

Plating efficiency appeared similar in all media as judged by the initial colony density. Therefore an estimate of growth was obtained from the surface area occupied by the cultures, with due allowance for the greater surface area occupied by differentiated cells:-

Arrested - no increase in the size of individual colonies after Day 4
Poor - very sparse cultures but some progressive growth
Intermediate - cultures semi-confluent by Day 7
Rapid - cultures confluent (>$5x10^5$cells/cm^2) by Day 7

Medium conditioned in the absence of serum and subsequently supplemented with 10% (v/v) foetal calf serum has similar effects as medium conditioned in the presence of 10% serum. This indicates that conditioning does not involve any direct interaction between BRL cells and serum components. Both foetal and newborn calf sera (10%) support growth in BRL-conditioned medium, but spontaneous differentiation appears more extensive in the latter at eqivalent dilutions of the conditioned medium. Moreover, a typical EC morphology of tightly clustered epithelial cells is displayed in the presence of foetal serum, whereas in newborn serum the cells assume a more spread configuration and the nuclei become more distinct, similar to the initial morphological changes during differentiation. The presence of 2-mercaptoethanol is essential for survival of PSA4 and its derivatives in BRL-conditioned medium (see Chapter 7). Consequently CM containing 10% foetal calf serum and 10^{-4}M 2-mercaptoethanol was employed in subsequent experiments. A standard culture medium of 60% (v/v) BRL-conditioned CM:40% normal CM was adopted for PSA4 and its derivatives. This will henceforth be referred to as "BRL-medium". Feeder-free cultures of PT2 have been maintained for 6 serial passages in such medium with no apparent deterioration in growth rate or increase in differentiation.

5.3. CHARACTERISATION OF THE EFFECTS OF BRL-CONDITIONED MEDIUM

5.3.1. Introduction

The discovery that PT2 cells retain EC morphology and growth behaviour in the absence of feeder cells when cultured in medium conditioned by incubation with BRL cells raises several important issues. Is this effect generally applicable to feeder-dependent EC and EK cells? Does it operate via inhibition of differentiation or by selection of a nullipotent subpopulation? Is the effect fully reversible or does the developmental potential of EC cells become permanently restricted by culture in this medium? Does BRL-medium prevent differentiation induced by other stimuli such as aggregation or exposure to retinoic acid? Conversely, can other stimuli overcome the block imposed by BRL-medium? These questions are addressed below. The answers have major implications for the application of BRL-conditioned medium to the *in vitro* maintenance of embryonic cells.

5.3.2. Applicability of BRL-Conditioned Medium to Feeder-Free Culture of Other Feeder-Dependent Embryonal Carcinoma and Embryo-Derived Stem Cells

Initial observations on the effects of BRL-medium were made on the EC cell line PT2, a subclone of PSA4TG12 (Slack *et al*, 1977). PT2 bears a close phenotypic resemblance to its parent line and is both feeder-dependent and pluripotential (Smith, 1984; see also Section 4.5.). Nonetheless, it is possible that it may possess hitherto unidentified variant properties. It was therefore considered important to determine whether BRL-conditioned medium could maintain other EC lines in the undifferentiated state in the absence of feeders.

Observations on PSA4TG12 and the original isolate PSA4 (Martin and Evans, 1975a), both in plating tests and in mass cultures, quickly established that these cell lines respond to BRL-medium in the same manner as PT2. Subsequent investigations into the effects of BRL-medium of feeder-dependent EC cells were made on PSA4. This is a pluripotential EC clone isolated from a teratocarcinoma, OTT-5568, derived from a 3 day embryo (Stevens, 1970). It differentiates extensively in tumours and in embryoid bodies. PSA4 also undergoes rapid differentiation when plated in the absence of feeders in normal medium containing 10^{-4}M 2-mercaptoethanol (Plate 5.2A). This latter form of differentiation does not occur in BRL-medium in which PSA4 proliferates as homogeneous embryonal carcinoma population (Plate 5.2B)

Provided the pH balance is maintained, growth of PSA4 in BRL-medium is accompanied by very little cell death. It is advisable to feed the cultures with fresh medium every third day to prevent significant levels of spontaneous differentiation. BRL-conditioned CM is quite stable to freezing and thawing, however, and can be stored at -20°C (Table 5.2), facilitating the routine maintenance of EC stocks in BRL-medium. Some differentiation does occur in BRL-medium if the EC cells become clumped and and form aggregates. It is therefore necessary to ensure that single cell suspensions are generated during subculturing, if necessary by passing the cells through a syringe needle (Section 2.3.1.).

Under the above conditions feeder-free cultures of PSA4 have been maintained for 10 serial passages, with a split ratio of 1 in 10 at each subculture. During this period there appeared to be no decrease in growth rate and differentiation remained negligible.

S2 is another feeder-dependent EC line derived from OTT-5568 (Martin and Evans, 1975a). It differs from PSA4 in that it only forms simple embryoid bodies which do not cavitate or undergo any further development than the delamination of endoderm while maintained in suspension (Martin and Evans, 1975a,b). S2 does differentiate when plated in the absence of feeders, however, and as with PSA4 and its derivatives, this differentiation does not proceed in BRL-medium.

Thus BRL-medium prevents the spontaneous differentiation of two independently isolated feeder-dependent EC lines. Furthermore, evidence will be presented below (Section 5.3.5.). that BRL-medium reduces retinoic acid-induced differentiation of the feeder-independent line PC13.5, derived from a different teratocarcinoma, OTT-6050 (Bernstine *et al*, 1973). It therefore appears that the effects of BRL-medium may be applicable to many, if not all, EC cells. Pluripotent embryonic stem cell lines have recently been established directly from mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981; Robertson *et al*, 1983; Axelrod, 1984). These EK cells closely resemble EC cells (see Section 1.6.). Unlike most EC lines, however, EK cells generally have euploid karyotypes. They also give greater contributions to chimaeras, including in some cases colonisation of the germ line at relatively high frequency (Bradley *et al*, 1984). Consequently, they represent a considerable advance in mammalian embryology.

EK cells are isolated on feeder layers and remain feeder-dependent, differentiating rapidly when plated on gelatinized substrata in the presence of 2-mercaptoethanol (Martin, 1981; Doetschman *et al*, 1985). The diploid EK line B2B2 (Robertson *et al*, 1983) is routinely maintained on STO feeders. It differentiates extensively when plated in the absence of feeders (Plate 5.4A). This differentiation is prevented by BRL-conditioned medium (Plate 5.4B). In 60-70% (v/v) BRL-conditioned medium B2B2 proliferates as a homogeneous stem cell population with less than 5% of cells showing signs of morphological differentiation. Such feeder-free cultures have been maintained for over a month (6 serial passages) with no change in growth rate or differentiation.

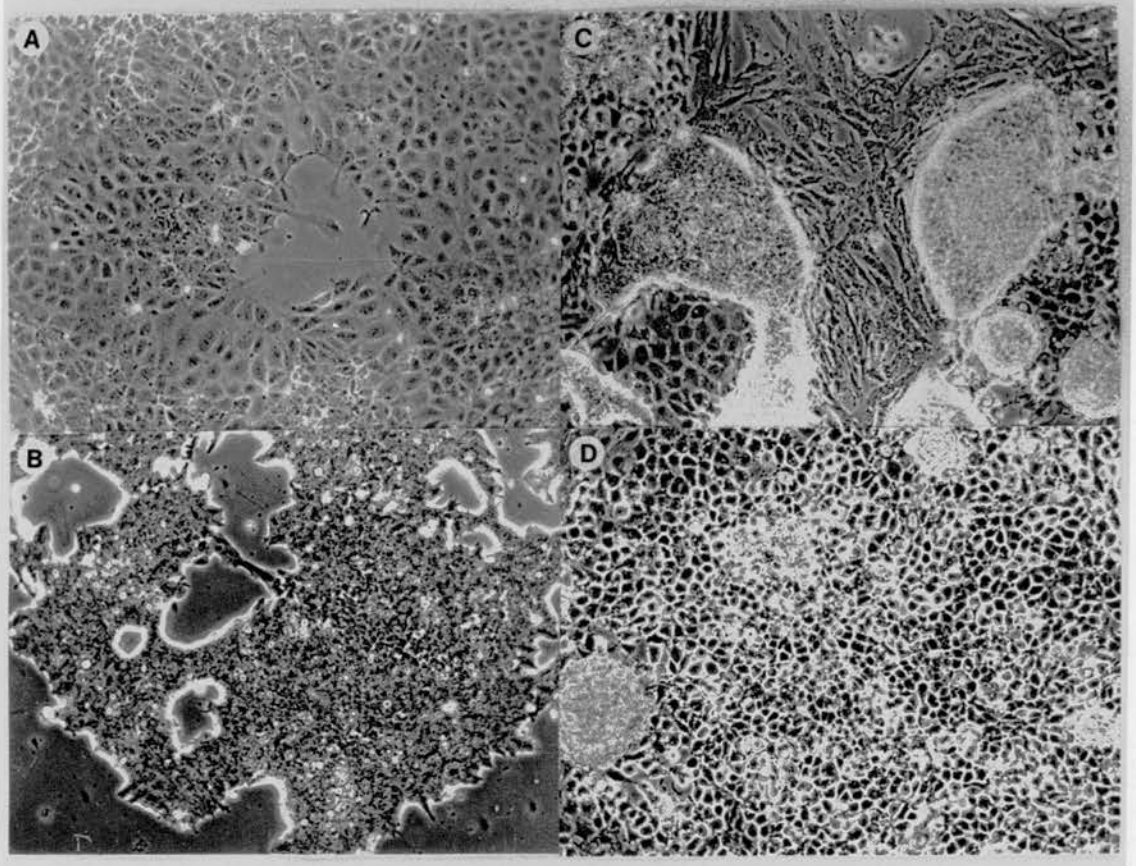
Plate 5.2 Feeder-Free Culture of PSA4 in BRL-Medium and Retinoic Acid-Induced Differentiation

Phase contrast

	Magnification
A. CM, 4 days	82
B. BRL-medium, third passage	98
C. BRL-medium plus 10^{-5} M retinoic acid, 24 hours, followed by 5 days in BRL-medium in the absence of retinoic acid	90
D. As C	98

Plate 5.3 PYS Parietal Endoderm Cell Line

Phase contrast
Magnification: 98


A
C
B
D

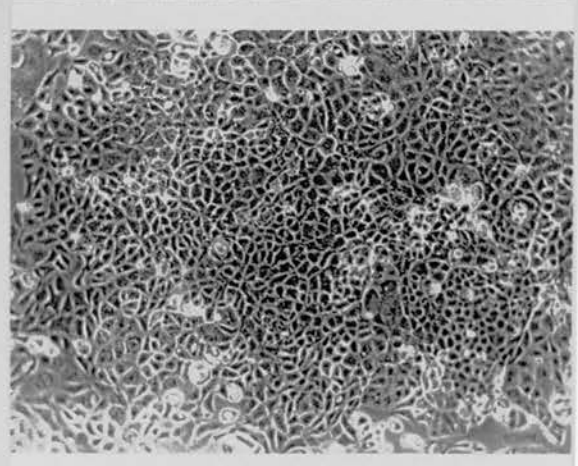
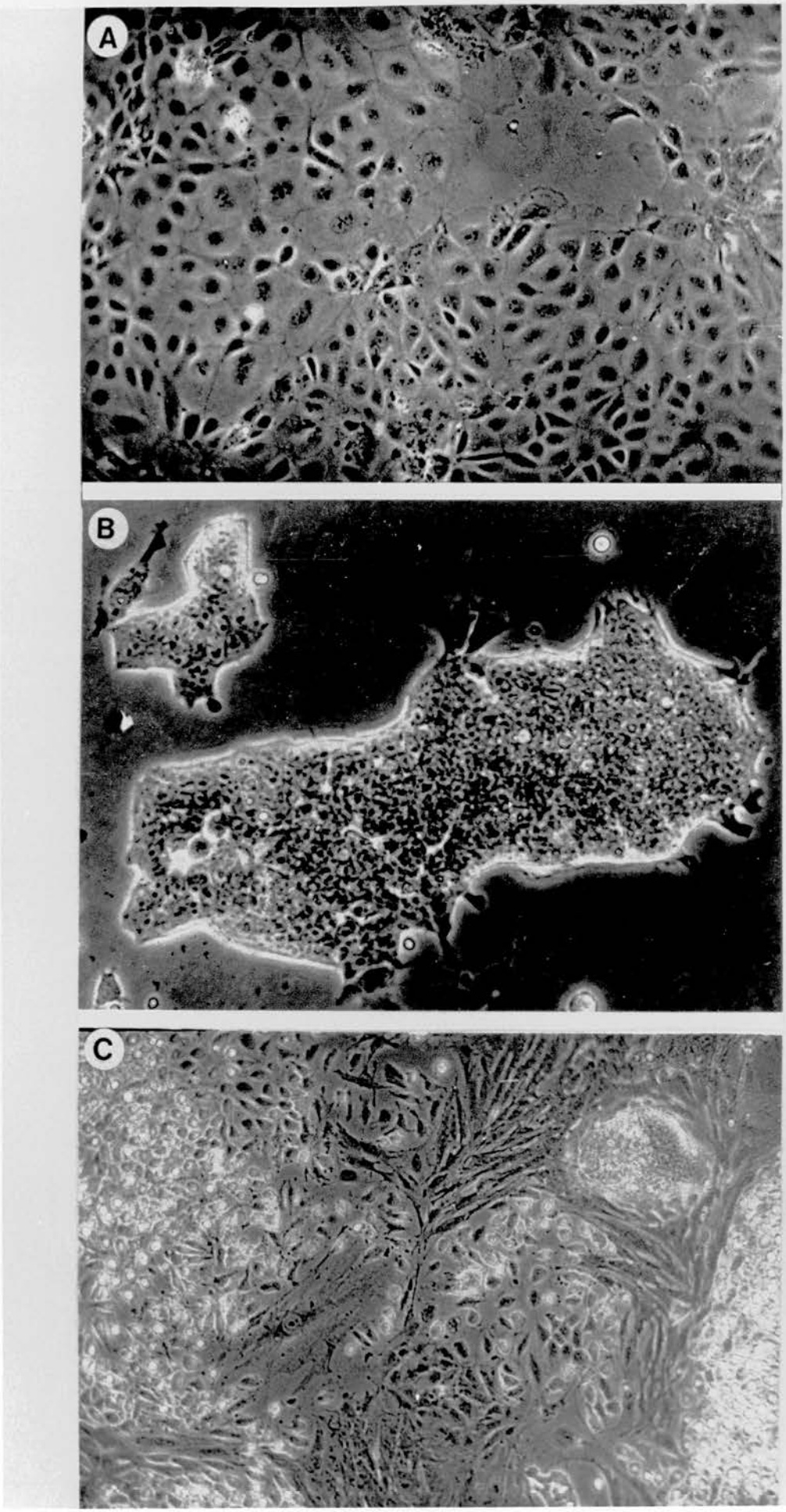


Plate 5.4 Feeder-Free Culture of the EK Cell line B2B2 in CM and in BRL-Conditioned Medium in the Absence and Presence of Retinoic Acid

Phase contrast

	Magnification
A. CM, 4 days	147
B. 70% (v/v) BRL-conditioned medium, passage 3	540
C. 70% (v/v) BRL-conditioned medium plus 10^{-7} M retinoic acid, 3 days, followed by 1 day in 70% (v/v) BRL-conditioned medium in the absence of retinoic acid	98


A
B
C

5.3.3. BRL-Medium Acts Via Reversible Inhibition of Differentiation

The observation that feeder-dependent EC and EK cells can be maintained in the absence of feeders in BRL-conditioned medium could be explained either by selection against, or inhibition of, differentiation. The former mode of action seems unlikely given the enhanced cloning efficiency and low level of cell death associated with culture in BRL-conditioned medium (Section 5.2). Moreover, there is no evidence of significant heterogeneity within cultures of PSA4, S2 or B2B2. On the contrary, PSA4TG12, a thiguanine resistant derivative of PSA4, and its unselected subclone PT2 provide a series of pluripotent subclones which respond to BRL-medium in the same manner as the parental line PSA4. Furthermore, in plating tests in the absence of feeders, 99% of PSA4 and B2B2 colonies (the great majority derived from single cells, Section 2.9.) in CM consist wholly or partly of differentiated cells (see Table 5.1).

As the number of PSA4 colonies formed in the absence of feeders is less than 40% of the number of cells seeded, there is a formal possibility that a nullipotent subpopulation exists which is not viable in the absence of feeders in CM (even in the presence of 2-mercaptoethanol). Hypothetically BRL-medium might both enable feeder-free growth of such a population and select against differentiating cells. This hypothesis is precluded by the data in Table 5.3 on the plating efficiency of PSA4 following culture in the absence of feeders.

Loss of colony-forming ability is an early event in the differentiation process (Jones-Villeneuve *et al*, 1982; Campione-Piccardo *et al*, 1985a) which has been used as an index of the commitment of EC cells (Rosenstraus and Levine, 1982; Campione-Piccardo *et al*, 1985a,b). The results in Table 5.3 indicate that over 98% of PSA4 cells differentiate or become committed to differentiate within 7 days of plating on gelatinized substrata in CM, even if they have previously been cultured in BRL-medium. Essentially all of the cells remain uncommitted in BRL-medium, providing further evidence of its capacity to support proliferation of a homogeneous stem cell population. (The low plating efficiency in Experiment II probably results from an uneven distribution of feeder cells over the substratum observed in this particular experiment.)

Table 5.3
Plating Efficiency of PSA4 on STO Feeder Layers After Feeder-Free Culture in CM or BRL-Medium

Experiment	Number of Colonies CM*	BRL*
I	13	1124
II	1	654
III	2	1252
IV	0.5	1290

*Cells previously passaged in normal CM (CM) or BRL-medium (BRL).

PSA4 cells previously passaged at least 3 times in BRL-medium in the absence of feeders were seeded at a density of $4x10^3/cm^2$ on gelatinized substrata in CM or BRL-medium. They were cultured for 7 days (6 days in Experiment I). Plating tests were then performed in duplicate on STO feeder layers as detailed in Section 2.9. Colony counts greater than 1000 can result from the formation of satellite colonies.

The finding that the effect of BRL-medium on spontaneous differentiation is readily reversible not only establishes that the process is one of inhibition rather than selection but also suggests that BRL-medium does not reprogramme EC cells and reduce their developmental potential. This issue is of crucial importance for any future applications of BRL-medium. Convincing evidence that no restrictions are imposed on future developmental capacity comes from induction of aggregation after extended periods of monolayer culture in BRL-medium. The aggregates rapidly delaminate an outer layer of endoderm and undergo cavitation at high frequency (Table 5.5). Occasionally the cystic embryoid bodies contract rhythmically, indicative of the presence of beating muscle. Histological sections reveal further differentiation in some of the cavitated embryoid bodies, including the formation of blood islands (Plate 5.6). When allowed to reattach to the substratum, outgrowths form which contain numerous diverse tissues and cell types, including muscle, cartilage, nerural tissue, pigmented cells and various epithelia (Plate 5.9). The differentiation potential of PSA4 thus appears unaffected by BRL-medium. In fact embryoid bodies formed after culture in BRL-medium show better development than is generally obtained with cultures taken directly from feeders layers, perhaps because residual feeders retard development in the

latter.

The karyotype of PSA4 also appears to be unaffected by prolonged culture in BRL-medium. After 10 serial passages the modal chromosome number remains 40 (range 38-40) with no gross abnormalities (Plate 5.7).

BRL-conditioned medium prevents EC cell growth unless diluted with normal medium (Section 5.2.). As some models of cytodifferentiation invoke an association of commitment with cell division (Levenson and Housman, 1981), it was considered important to determine the growth rate of PSA4 in BRL-medium. A comparison of the population growth of PSA4 on STO feeders, and in the absence of feeders in CM or BRL-medium is shown in Fig.5.1.

The rates of population increase are essentially identical for the first 4-5 days after plating. Growth on feeders then begins to level off as the cultures near confluence and rapidly acidify the medium, inducing considerable cell death. This occurs more quickly on feeders than on gelatin due to the three-fold higher initial plating efficiency on the former. Growth in normal CM in the absence of feeders also slows after 5 days (4 days in unmodified medium, since the first 24 hours after plating are in BRL-medium) coinciding with the onset of overt differentiation. The population in BRL-medium continues to increase, however, as the cultures do not approach confluence until Day 7 and differentiation is negligible.

As there is little cell death in any of the cultures over the first 4 days, the population growth rate in this period is a good reflection of EC cell generation time. The results therefore indicate that when diluted 60:40 with normal CM, BRL-conditioned medium neither reduces the rate of cell division of the global population, nor induces a significant subpopulation to withdraw completely from the cell cycle. Consequently it may be concluded that the effects of BRL-medium on EC cell differentiation are not mediated via inhibition of the cell cycle.

Finally the expression of three antigenic markers of embryonal carcinoma was investigated in cultures of PSA4 maintained in the absence of feeders in CM or in BRL-medium (Table 5.4).

Fig.5.1 Growth Curves for PSA4

Growth curves were determined as described in Section 2.8. on feeders (F) or on gelatinised substrata in BRL-medium (BRL) and in normal medium (CM). Each data point is the mean±s.e.m. obtained from a single experiment performed in triplicate.

Fig.5.1 GROWTH CURVES FOR PSA4

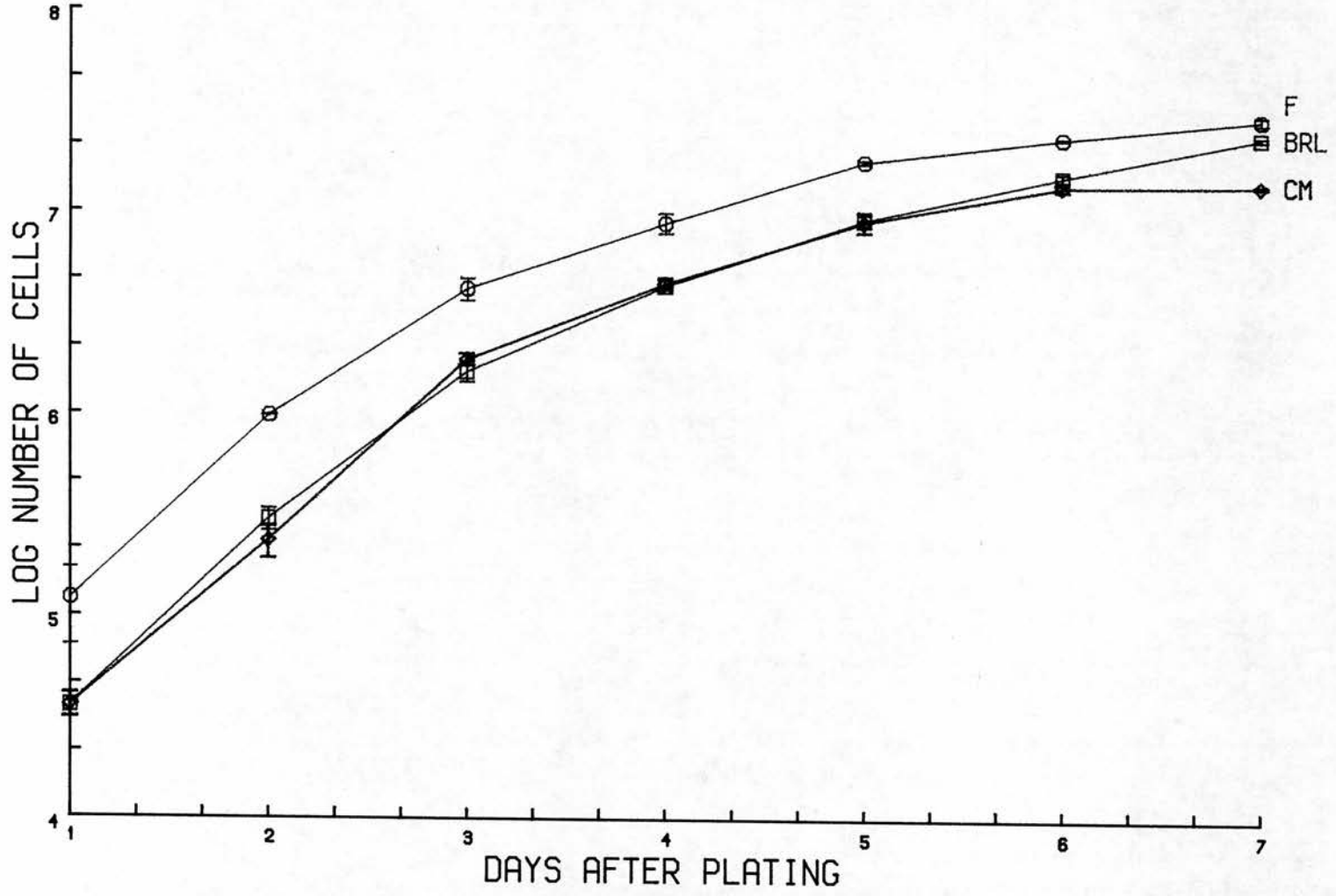


Table 5.4
Indirect Immunoperoxidase Staining Of PSA4 Cultured in CM or BRL-Medium

Antibody	Normal CM	BRL-Medium
Anti-SSEA-1	-	+++
M1/22-25	±-	++
2H9	±+	+++

Monolayers on Thermanox coverslips were fixed and assayed as described in Section 2.19. Estimates of the proportion of antigen-positive cells are:-

+++ over 90% of cells positive
++ 75-90% of cells positive
±+ over 80% of cells positive, but a morphologically distinct subpopulation (<10%) negative
±- over 80% of cells negative, but a morphologically distinct subpopulation (<10%) positive
- less than 5% positive

Two of the monoclonal antibodies, anti-SSEA-1 and M1/22-25, have been described above (Section 4.3.2.). The third, 2H9, is a rat IgG which detects a glycoprotein present on EC cells and also reacts with some differentiated cell types (Stern *et al*, 1984). Cells grown in BRL-medium gave a positive indirect immunoperoxidase reaction with all three antibodies. Not all cells in these apparently homogeneous populations reacted uniformly, but this is not uncommon for antibodies against embryonal carcinoma, as described earlier (Section 4.3.2). Cells cultured in CM gave no specific staining of their cell surfaces after incubation with anti-SSEA-1. A distinct subpopulation of the differentiated cells generated in CM did react with M1/22-25 (Plate 5.5A). These were small clusters of dispersed epithelioid cells, in marked contrast to the bulk of the cultures which consisted of a pavement of flattened, closely apposed cells. Conversely, 2H9 bound to most of the differentiated cells, but not to the occasional areas of epithelioid morphology (Plate 5.5B).

These results demonstrate that PSA4 cells retain certain antigens characteristic of undifferentiated embryonal carcinoma when cultured in the absence of feeders in BRL-medium, whereas changes in cell surface antigen expression accompany morphological differentiation in CM.

Plate 5.5 Indirect Immunoperoxidase-Staining of PSA4 Monolayers Following Spontaneous Differentiation in CM

Monolayers of PSA4, cultured in the absence of feeders in CM on Thermanox coverslips for 6 days, were fixed in acetone and processed for immunoperoxidase staining as described in Section 2.19. Primary antibodies are M1/22-25 (A) and 2H9 (B). Specimens are counter-stained with haemotoxylin.

	Magnification
A. M1/22-25, positive cell cluster arrowed	125
B. 2H9, non-staining cell cluster arrowed	125

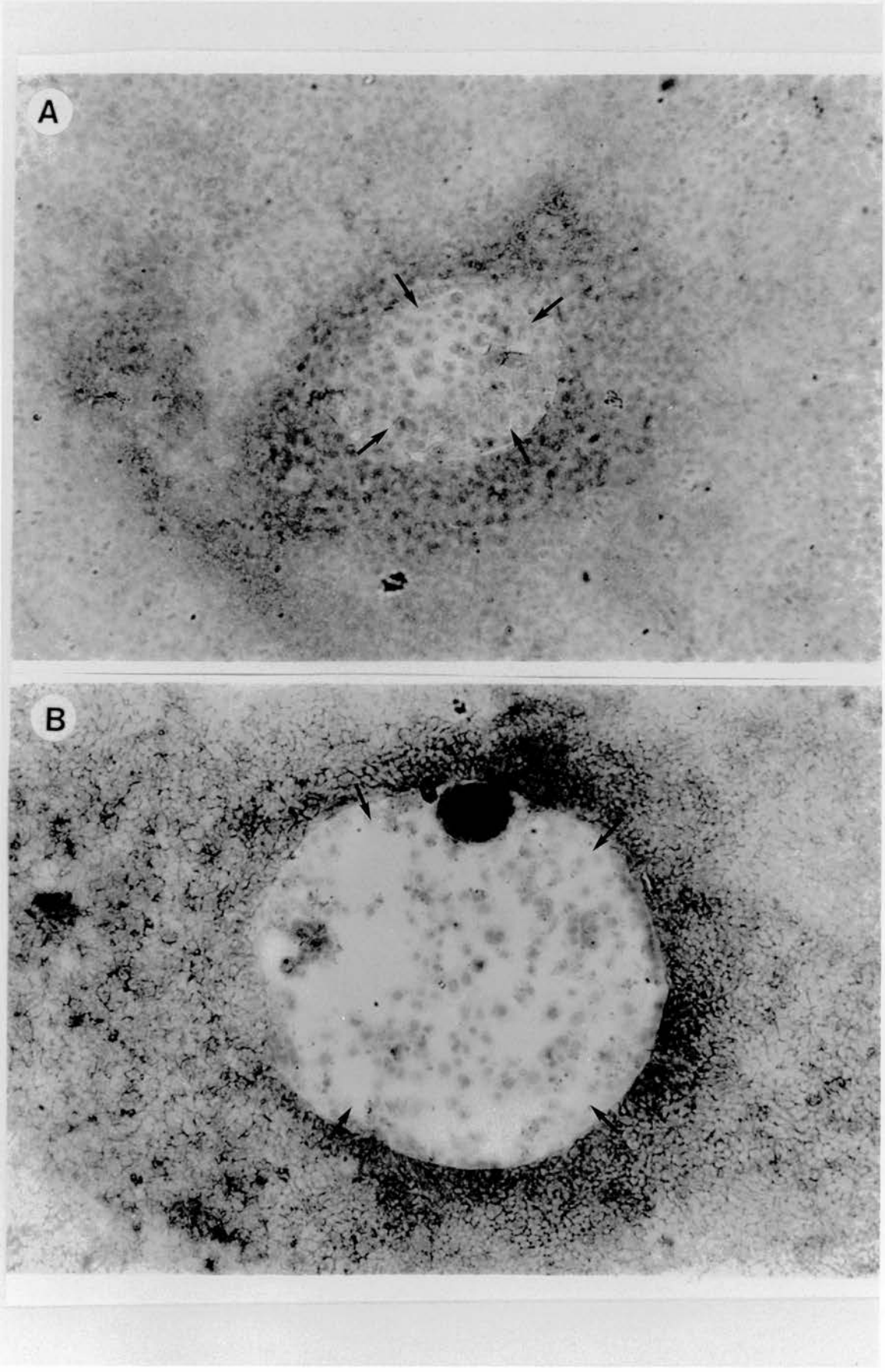

A
B

Plate 5.8 PSA4 Embryoid Bodies Formed in BRL-Conditioned Medium

PSA4 cells, previously passaged 4 times in BRL-medium, were induced to form embryoid bodies via aggregation (Section 2.10.3.) in 60:40 (v/v) BRL-conditioned EfC10:normal EfC10. Embryoid bodies were fixed in 96% ethanol:glacial acetic acid (99:1 v/v) at 4^0C after 6 days of suspension culture. Histological sections were stained with haemotoxyin and eosin (H+E) or with periodic acid Schiff (PAS).

	Magnification
A. Embryoid bodies, H+E	76
B. Embryoid bodies with region of endodermal proliferation (arrowed), H+E	67
C. Region of endodermal proliferation, PAS	231
D. Embryoid bodies with parietal endoderm-like outer layer (PE) on thick basement membrane (BM), H+E	231
E. Vacuolated visceral endoderm-like cells (VE) on thin basement membrane (BM), H+E	1051

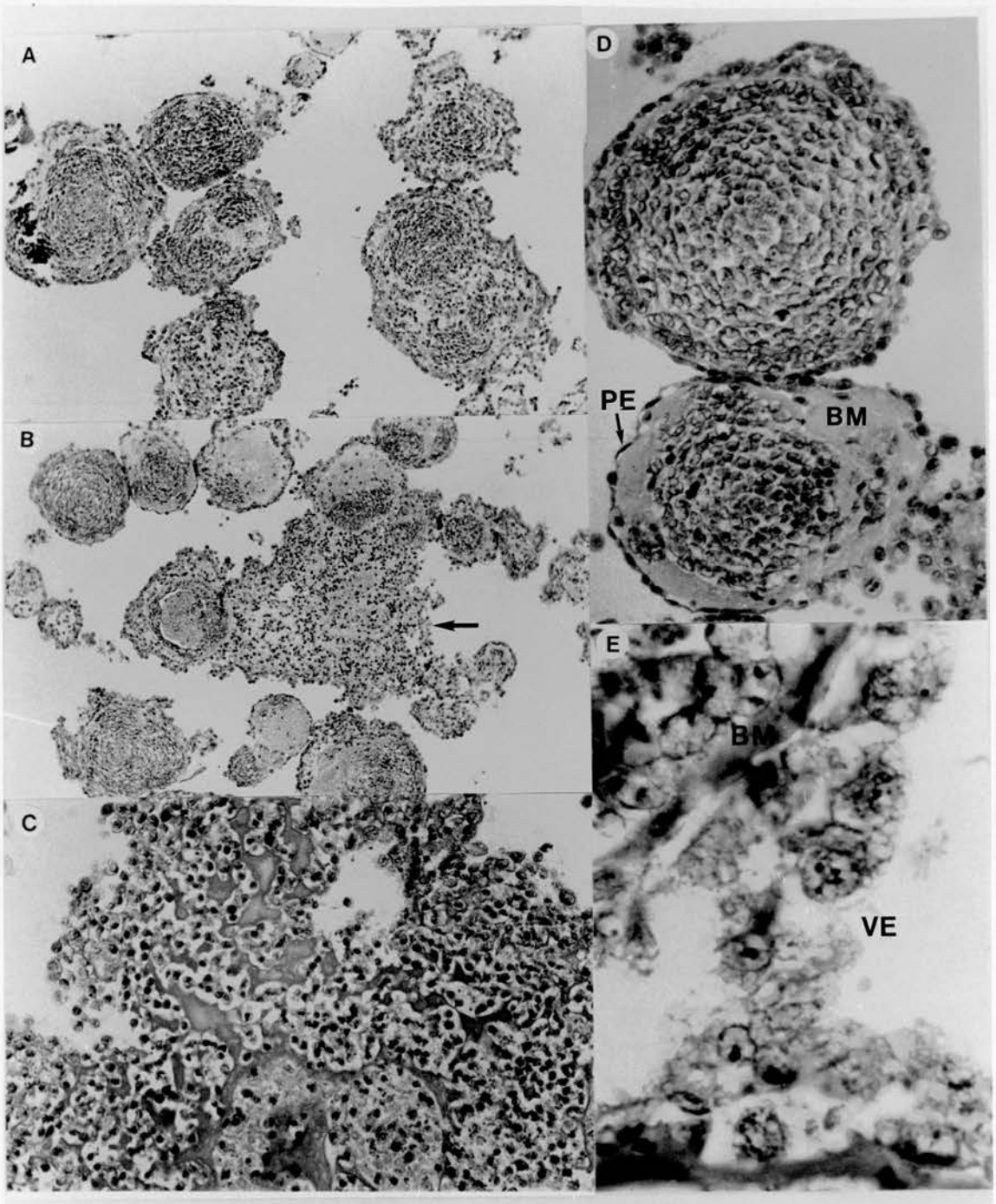

A
B
C
D
PE
BM
E
BM
VE

Plate 5.9 PSA4 Embryoid Body Outgrowths

PSA4 cells, previously passaged 4 times in BRL-medium, were induced to form embryoid bodies (Section 2.10.3.) in normal EfC10 (A-D) or in 60% (v/v) BRL-conditioned EfC10 (E-H). The embryoid bodies were allowed to attach to Thermanox coverslips in CM (Section 2.10.4.) and cultured for 14 days before fixation, histological processing and staining with haemotoxylin and eosin.

	Magnification
A. Differentiated outgrowth	76
B. Disorganised outgrowth of differentiated cells	79
C. Mixture of differentiated cell types, including putative trophoblast giant cell (T)	126
D. Various epithelial and pigmented (P) cells	126
E. Epithelium of unidentified cells	126
F. Fibroblastic outgrowth	60
G. Cartilage	182
H. Undifferentiated embryonal carcinoma (EC)	126

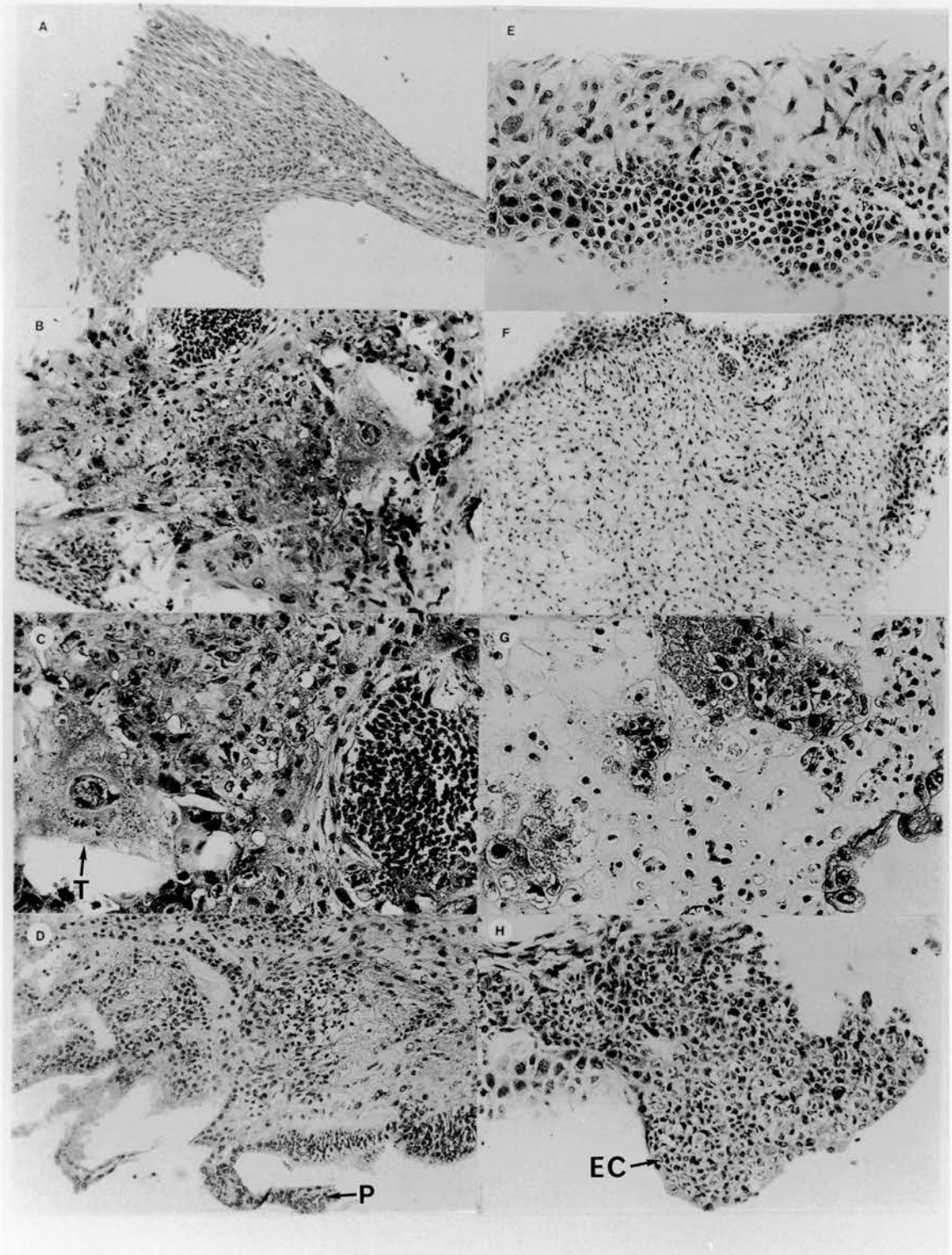
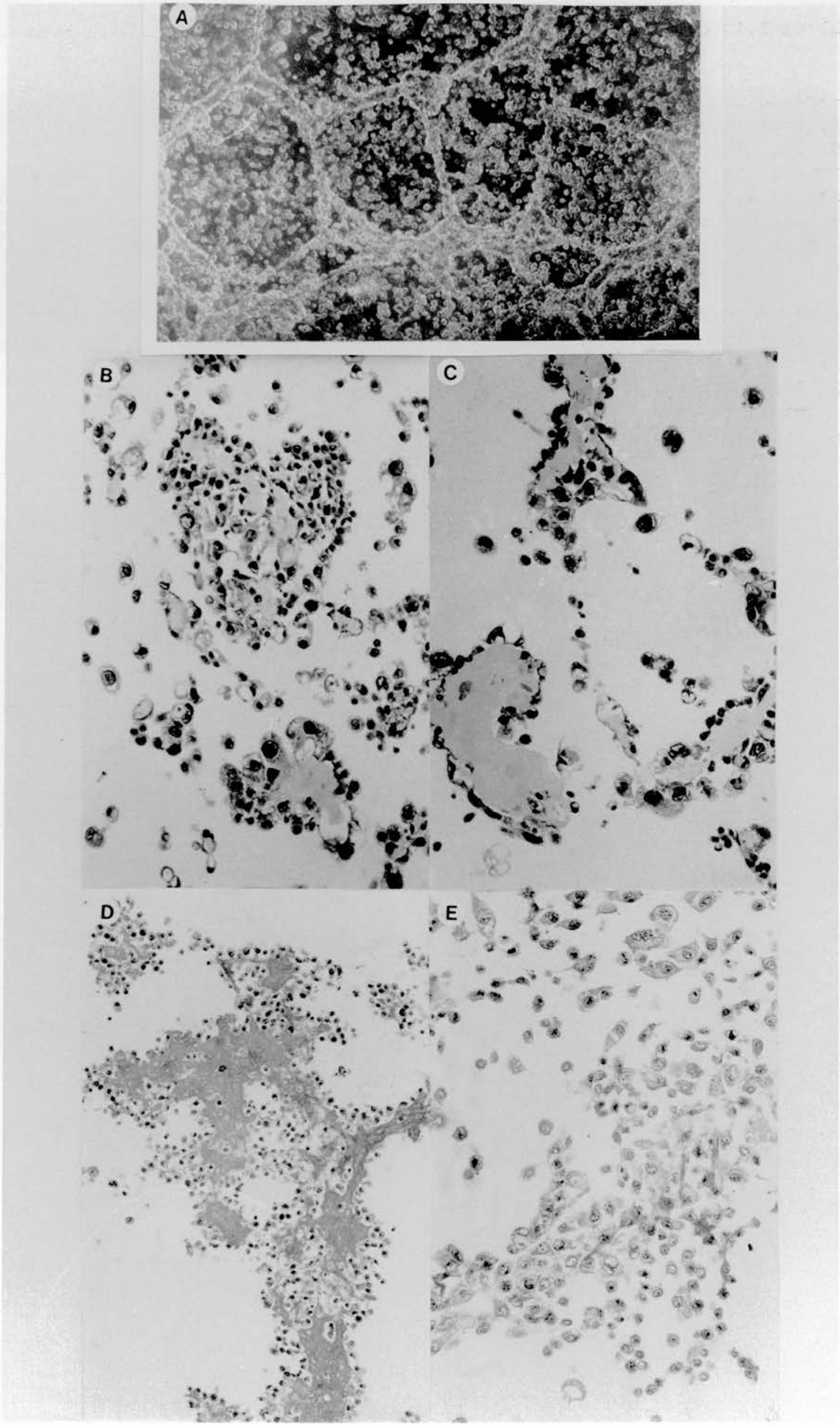

A
E
B
F
C
G
T
D
H
P
EC

Plate 5.10 Outgrowths from PSA4 Embryoid Bodies Formed and Maintained in BRL-Conditioned Medium

PSA4 cells, previously passaged 4 times in BRL-medium, were induced to form embryoid bodies (Section 2.10.3.) in 60% BRL-conditioned EfC10. The embryoid bodies were allowed to attach to Thermanox coverslips (Section 2.10.4.) in BRL-medium and cultured for 14 days before subculturing or fixing and processing. Passaged cells were maintained in BRL-medium and photographed (A) using dark field optics. Histological sections (B-E) were stained with haemotoxylin and eosin (H+E) or periodic acid Schiff (PAS).

	Magnification
A. Subcultured cells, 10 days post-trypsinization	43
B. Rounded, matrix-producing cells, H+E	172
C. " "	172
D. Ribbon of extracellular matrix and rounded cells, some embedded in matrix, PAS	69
E. Epithelioid and rounded cells, PAS	237


A
B
C
D
E

5.3.4. The Effect of BRL-Conditioned Medium on Differentiation in Aggregates

Feeder-dependent EC lines such as PSA4 can be induced to differentiate either by plating in the absence of feeders or via the formation of aggregates which subsequently develop into embryoid bodies (Section 1.4.). BRL-medium has been shown to inhibit the former differentiation into a monolayer of ill-defined cell types. The differentiation of embryoid bodies is more highly organised, resembling in many respects the development of the inner cell mass (ICM) of the pre- and early post-implantation mouse blastocyst (Martin, 1975; Martin *et al*, 1977). It thus provides a model of events in normal embryogenesis. Consequently the effects of BRL-conditioned medium on this developmental system are of considerable interest.

Data from a series of aggregation experiments are summarized in Table 5.5. The aggregates are classified according to Smith (1984) into three types: undifferentiated (A), simple embryoid bodies (B), and cystic embryoid bodies (C).

Table 5.5
Classification of PSA4 Aggregates Formed in Normal EfC10 and 60% (v/v) BRL-Conditioned EfC10 Medium

	Medium*		Days in	Aggregate Type			% Cystic
Expt.	1	2	Suspension	A	B	C	Aggregates
I	E	E	4	0	49	50	51
I	E	E	6	1	6	23	77
II	E	E	2	1	26	10	27
II	E	E	6	0	7	16	70
II	M	M	4	2	97	1	1
II	M	M	6	7	89	1	1
II	E	M	6	2	13	12	44
III	E	E	6	0	12	12	50
III	M	M	6	1	16	1	6

*1 and 2 refer to Stages 1 and 2 respectively of the aggregation protocol described in Section 2.10.3.
E,EfC10; M,60:40 (v/v) BRL-conditioned EfC10:normal EfC10

In all cases PSA4 cells had previously been maintained in BRL-medium in the absence of feeders for at least four serial passages. Aggregates were classified as follows:-

A. Undifferentiated EC aggregate.
B. Simple embryoid body; EC core surrounded by a layer of matrix-producing endodermal cells on a basement membrane.
C. Cystic embryoid body; cavitated embryoid body, usually containing EC cells and often various differentiated cells.

It is apparent that in normal EfC10 medium the majority of embryoid bodies undergo cavitation and this is often accompanied by further differentiation (Plate 5.6). In contrast, aggregates maintained in 60% BRL-conditioned EfC10 delaminate an outer layer of endoderm but apparently undergo no further development (Plate 5.8). These simple embryoid bodies contain varying proportions of endoderm, basement membrane, extracellular matrix and EC cells, but other differentiated cell types are never present. Areas of necrosis can sometimes be identified. Both the basement membrane and the matrix material stain intensely with periodic acid Schiff (PAS) (Plate 5.8C). This is diagnostic of the presence of polysaccharide and suppports the identification of the outer layer of cells as extraembryonic endoderm (Martin and Evans, 1975b), since Reichert's membrane in the implantation stage blastocyst likewise stains with PAS. Reichert's membrane is produced by parietal endoderm (Section 1.2.2.). Some of the columnar endodermal cells are underlaid by a thin basement membrane and their apical cytoplasm is vacuolated (Plate 5.8E), features consistent with the visceral phenotype. Morphological evidence alone cannot be considered diagnostic, however, and confirmation that more than one type of endoderm is present would require ultrastructural analyses (Martin *et al*, 1977) and/or immunological detection of specific markers, for example alphafoetoprotein or transferrin which are produced by visceral but not by primitive or parietal endoderm (Adamson, 1982) (see Section 1.2.2.).

BRL-conditioned medium thus permits the differentiation of endoderm in EC aggregates, but prevents the developmental transition from simple embryoid body to complex embryoid body.

In one experiment in which the initial aggregation (Stage 1) was performed in normal EfC10 and the suspension stage (Stage 2) in 60% BRL-conditioned EfC10, an intermediate number of cystic embryoid bodies were generated (Table 5.5). These embryoid bodies displayed a similar level of complexity to cystic structures obtained following continuous maintenance in normal EfC10. This indicates that, once initiated, cavitation may proceed normally in BRL-conditioned medium. The implication is that BRL-conditioned medium does not select against differentiating embryoid bodies but rather that it prevents the onset of cavitation and/or further differentiation.

Greater amounts of endoderm and extracellular matrix are produced after aggregation in BRL-conditioned as compared with normal EfC10. This may be

related to the failure of the embryoid bodies to cavitate. However, the occurrence of large regions of endodermal cells (Plate 5.8B,C) suggests that whilst aggregation triggers their differentiation, BRL-conditioned medium may directly promote their proliferation. This is supported by observations on outgrowths formed after the embryoid bodies are allowed to reattach (see below). A direct effect of BRL-conditioned medium on endoderm differentiation cannot be precluded of course, but it is notable that large numbers of EC cells persist in many of the aggregates but are encased by basement membrane (Plate 5.8A,D), suggesting that they do not contribute to further development of the endoderm.

The restricted development of embryoid bodies in BRL-conditioned medium does not appear to limit differentiation potential on reattachment to the substratum. If attachment is in CM, outgrowths develop which contain a variety of tissues and cell types, including beating muscle, cartilage, various epithelia, endodermal cells, fibroblasts and some undifferentiated cells (Plate 5.9). This differentiation is similar to, though not as extensive as, that found in outgrowths from embryoid bodies formed in unmodified EfC10. However, if attachment is in BRL-medium rather than in CM, the outgrowths all appear to be of a single form composed of two types of cell, one flattened and epithelioid, the other rounded (Plate 5.10). Interestingly EC cells have not been observed. The outgrowths produce copious amounts of a PAS-positive extracellular matrix and the rounded cells become embedded in ribbons of this material. Both flattened and rounded cells stain weakly with PAS. These cells are capable of at least limited proliferation in BRL-medium and have been subcultured for up to three serial passages, though attempts to clone them have proved unsuccessful. After subculturing, the cultures initially consist solely of the epithelioid cells which show some morphological similarities with the parietal yolk sac line PYS (Lehman *et al*, 1974). Subsequently the rounded cells appear and progressively take over the culture. It is possible that the rounded cells are a differentiation product of the flattened cells, perhaps representing the transition from primitive to parietal endoderm (see Section 1.2.2.). Parietal endoderm cells tend to adopt a rounded configuration *in vitro* (Enders *et al*, 1978; Gardner, 1983a). Alternatively they may be a distinct population which require the flattened cells for multiplication via a feeder effect. Further characterisation and hopefully identification of these cell types is necessary to resolve this issue. Such an investigation might also detect

other differentiated tissues and cell types which could be present in small amounts. Again it should be noted that the effect of BRL-medium is not mediated via cell selection since differentiated outgrowths initiated in CM survive and continue to develop if transferred to BRL-medium. However, the effect appears to be irreversible at this stage since outgrowths initiated in BRL-medium do not show any further differentiation if transferred to CM. This is presumably because any remaining EC cells differentiate or die shortly after reattachment and only the endodermal derivatives persist. This would also account for the absence of outgrowths of EC cells in BRL-medium.

5.3.5. The Effects of BRL-Conditioned Medium on The Induction of Differentiation of Feeder-Independent PC13.5 EC Cells by Retinoic Acid

It has been shown above that BRL-conditioned medium inhibits differentiation of feeder-dependent EC cells induced by plating in the absence of feeders (Sections 5.2. and 5.3.2.) and restricts the development of embryoid bodies induced by aggregation of EC cells (Section 5.3.4). A third stimulus for differentiation is exposure to retinoic acid (Strickland and Mahdavi, 1978). This chemical induces differentiation of all feeder-independent EC lines tested with the exception of a handful of variants specifically selected for non-responsiveness (see Sections 4.3. and 4.6.). Therefore the effect of BRL-conditioned medium on the response of PC13.5 EC cells to retinoic acid was investigated.

PC13.5 (Hooper and Slack, 1977) is a clone of the feeder-independent EC line PC13 (Bernstine *et al*, 1973) which is derived from teratocarcinoma OTT-6050. It displays negligible levels of spontaneous differentiation in monolayer culture, but differentiates readily when treated with retinoic acid (Adamson *et al*, 1979). Initial observations on mass cultures indicated that retinoic acid does induce differentiation of PC13.5 in BRL-medium, but that such differentiation is less extensive than that induced by equivalent concentrations of retinoic acid in CM. The flattened differentiated cells produce large amounts of plasminogen activator. Therefore the fibrin overlay assay (Section 2.10.5.) was employed to obtain a quantitative comparison of the differentiation responses of PC13.5 to retinoic acid in CM or in BRL-medium (Table 5.6).

Table 5.6
Fibrin Overlay Assays for Plasminogen Activator Production by Retinoic Acid-Treated PC13.5 Cells

		%Colonies Inducing Lysis			
	RA*	Normal CM	BRL-Medium	100% BRL^	R~
Expt.1	10^{-7}	85.4	42.5	7.7	2.01
Expt.2	10^{-7}	77.5	46.7	-	1.66
Expt.3	10^{-7}	51.8	17.3	-	2.99
"	10^{-6}	-	32.3	-	-

*Concentration of retinoic acid (M)
^Undiluted BRL-conditioned medium
~R= Colonies Inducing Lysis in Normal CM / Colonies Inducing Lysis in BRL-Medium

Cultures were established as detailed in Section 2.9. and incubated with retinoic acid for 5 days, with a change of medium on Day 3. Assays were performed as described in Section 2.10.5. on duplicate dishes each containing over 200 colonies. All lysis was dependent on the presence of plasminogen and less than 1% of colonies incubated in medium supplemented with DMSO alone induced lysis.

The data show that BRL-medium causes an approximately 2-fold reduction in the differentiation response to 10^{-7}M retinoic acid. This is not due to an effect on cell growth as colonies in BRL-medium are comparable in size to those in CM. Nor does it arise from a change in the type of differentiated cell obtained or merely from inhibition of plasminogen activator production, since morphological assessments indicate that many colonies in BRL-medium are composed purely of EC cells whereas virtually all colonies in CM display signs of overt differentiation. Undiluted BRL-conditioned medium apparently causes a much greater reduction in retinoic acid-induced differentiation. However, growth is inhibited in this medium (see Section 5.2.) which may prevent cell commitment. The smaller colony size also increases the amount of plasminogen activator production per cell required for detection in this assay.

From morphological observations on mass cultures it is apparent that in BRL-medium as in normal CM, the proportion of EC cells which differentiate increases with increasing retinoic acid concentration. A given differentiation response appears to requires a 10-100-fold higher level of retinoic acid in the latter, however. This is unlikely to be due to inactivation of the retinoid since it remains active after incubation with BRL cells (Pitts *et al*, in press) and retinoic acid-induced differentiation of PSA4TG12 in BRL-medium is detectable at concentrations as low as 10^{-9}M (Section 3.2.1.). Consequently it seems reasonable to conclude that BRL-medium inhibits differentiation of EC cells

induced by retinoic acid. A similar effect has been reported for medium conditioned by STO feeder layers (Smith and Hooper, 1983). This inhibition is only be partial, however. This may be because retinoic acid is a more potent inducer of differentiation than other stimuli, as discussed in Section 4,5.4.

5.3.6. The Effects of Retinoic Acid on Feeder-Dependent EC Cells Cultured in BRL-Medium

The discovery that BRL-medium prevents the spontaneous differentiation of feeder-dependent EC cells plated in the absence of feeders (Sections 5.2. and 5.3.2.) makes it possible to study the effects of retinoic acid on pure homogeneous populations of these cells. Some of the results have been presented in preceding Chapters (Sections 3.2.1, 3.2.2. and 4.5.3.).

It was initially observed that exposure of feeder-free mass cultures of PSA4 in BRL-medium to 10^{-5}M retinoic acid for 24 hours resulted in morphological differentiation of the vast majority of the population. The differentiated cells obtained are markedly different from the pavements of flattened, closely apposed cells generated by spontaneous differentiation in CM (Plate 5.2A). They are predominantly refractile epithelioid cells, though some large fibroblastic cells are also present (Plate 5.2C,D). These epithelioid cells resemble PYS parietal endoderm cells (Plate 5.3) and some of the presumptive endoderm cells present in outgrowths from embryoid bodies maintained in BRL-conditioned medium (Section 5.3.4.). Like the latter they are capable of at least limited proliferation, rapidly outgrowing the fibroblastic cells which appear incapable of multiplication. Relatively pure populations have been maintained for three consecutive passages. Attempts at cloning have proved unsuccessful, however, possibly because these cells do in fact have a limited proliferative lifespan. Their morphology is unaffected by removing retinoic acid or changing the medium to CM, but only BRL-medium supports growth at low density. At high density considerable amounts of extracellular matrix material are deposited on the substratum, though not as much as is produced by embryoid body outgrowths.

The epithelioid cells release large amounts of plasminogen activator (Table 5.7; see also Section 3.2.1.).

Table 5.7
Fibrin Overlay Assay for Plasminogen Activator Production by PSA4

Medium	RA*	Number of Colonies	Lytic Zones	%Colonies Producing Plasminogen Activator
CM^	-	156	2.5	1.6
BRL	-	369	6.0	1.6
BRL	+	259	130.0	50.0

*10^{-7}M retinoic acid, 24 hour exposure
^The effect of retinoic acid in CM cannot be assayed under these conditions due to its high toxicity (Section 3.2.2.).
Assays were performed as described in Section 2.10.5. on duplicate cultures 4 days after exposure to retinoic acid. Lysis was dependent on the presence of plasminogen.

The data in Section 3.2.1. show that the induction of plasminogen activator is related to the concentration of retinoic acid (Fig.3.1), supporting the morphological evidence that the differentiation response is dose-dependent.

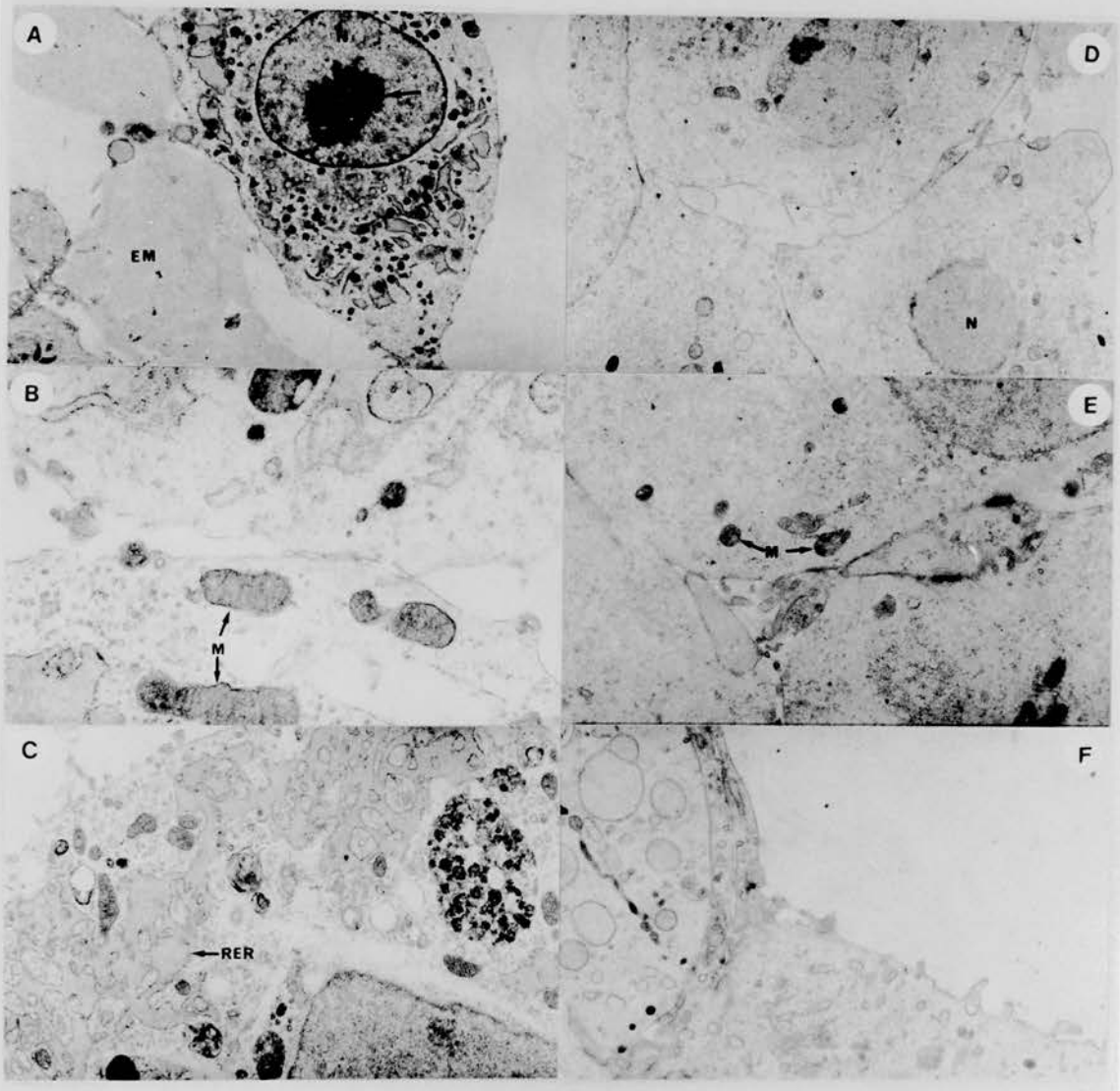
The production of plasminogen activator marks a biochemical distinction between the cells generated by retinoic acid treatment in BRL-medium and those produced via spontaneous differentiation in CM. In the experiments on PSA4TG12 and PTmr0 reported in Section 3.2.1. it was likewise found that the two types of differentiated cell differed in their production of this enzyme. The tissue activator form of plasminogen activator, which is more active in this assay than the urokinase form (Bachmann and Kruithof, 1984), is produced in large amounts by parietal endoderm (Marotti *et al*, 1982). The very occasional areas of lysis produced by cultures not exposed to retinoic acid, either in CM or in BRL-medium, are invariably associated with the presence of a small group of epithelioid cells. These cells are similar in morphology to those induced by retinoic acid in BRL-medium. Those obtained in CM are probably identical to the subpopulation described in Section 5.3.2. (Table 5.4), which express Forssman antigen, known to be present on extraembryonic endoderm (Willison and Stern, 1978; Evans *et al*, 1979), but do not bind 2H9. The few differentiated cells which sometimes appear spontaneously in BRL-medium, usually associated with aggregated clumps of EC cells, share this epithelioid morphology and may be of the same type.

The differentiated cells derived by these two treatments are also dissimilar at the ultrastructural level (Plate 5.11.). The retinoic acid-induced cells contain abundant cell organelles and exhibit distended profiles of rough endoplasmic reticulum, often filled with secretory material. The cells are often associated with regions of extracellular matrix. They display few surface microvilli and

Plate 5.11 Electron Micrographs of Differentiated Cells Produced by Retinoic Acid Treatment of by Spontaneous Differentiation in the Absence of Feeders

Monolayer cultures of B2B2 in 70% (v/v) BRL-conditioned medium plus 10^{-7}M retinoic acid (A-C) or PSA4 in CM (D-F) were maintained on Permanox dishes for 6 days, then fixed and processed for electron microscopy (Section 2.5.).

	Magnification
A. Retinoic acid-induced B2B2 derivative and area of extracellular matrix (EM). Note prominent nucleolous (arrowed) in the nucleus (N).	2,800
B. Short region of membrane apposition between adjacent retinoic acid-treated B2B2 cells containing numerous mitochondria (M).	15,000
C. Cytoplasm of retinoic acid-treated B2B2 cell exhibiting abundant cell organelles and cisternae of rough endoplasmic reticulum (RER) filled with amorphous matrix material.	6,200
D. Pavement of closely apposed, flattened PSA4 derivatives generated by spontaneous differentiation in CM.	3,500
E. Cytoplasm, with mitochondria (M), and cell boundaries of spontaneously differentiated derivative of PSA4.	5,600
F. Cell surface and rare region of vacuolated cytoplasm of spontaneously differentiated PSA4 derivative.	7,000


A
EM
B
M
C
RER
D
N
E
M
F

adjacent cells form intercellular contacts discontinuously. These observations are consistent with their being parietal endoderm (Martin *et al*,1977; Hogan *et al*, 1981). Spontaneously differentiated cells contain fewer subcellular organelles, have relatively little endoplasmic reticulum and show no signs of matrix production. Nor do they possess the numerous surface microvilli and electron-lucent vacuoles diagnostic of visceral endoderm (Martin *et al*, 1977). They appear as sheets of flattened cells in continuous intercellular contact with no indications of great synthetic or proliferative activity.

The above results demonstrate that retinoic acid can induce differentiation of feeder-dependent EC cells. Of greater significance is the observation that these findings apply equally to the EK line B2B2. As for PSA4, exposure of feeder-free cultures of B2B2 in BRL-medium to retinoic acid causes differentiation into parietal endoderm-like cells (Plates 5.4 and 5.11). Since all EK lines are feeder-dependent, this is the first unequivocal demonstration that they can be induced to differentiate by retinoic acid. This is significant as it provides good evidence that responsiveness to retinoic acid is not a consequence of the transformed phenotype of EC cells but is a property of the pluripotent cells of the embryonic inner cell mass (but see Section 5.4.).

The retinoic acid-induced differentiation of F9 EC cells into parietal endoderm is enhanced by dibutyryl cyclic AMP (Strickland and Sawey, 1980, Hogan *et al*, 1981). The growth and morphology of PSA4 cultures in BRL-medium are unaffected by dibutyryl cyclic AMP (10^{-3}M). Nor does it appear to have any effect on the type of differentiated cell obtained on exposure to retinoic acid. This indicates that the retinoic acid-induced processes of commitment and differentiation in BRL-medium are executed in full without requirement for any additional factors.

An alternative inducing agent, hexamethylenebisacetamide (HMBA) (Speers *et al*, 1979; Nicolas *et al*, 1981), is shown in Section 4.5.3. to promote the differentiation of PSA4-derived EC cells in CM. However, at the same concentration (3mM) this chemical has no obvious effects on PSA4 cultures in BRL-medium. This may reflect a difference in the mechanisms by which retinoic acid and HMBA induce differentiation (see Section 4.5.4), a difference in their relative potencies as inducing agents, or possibly an effect of the serum. The latter is conceivable because the induction of PT2 differentiation reported in Section 4.5.3 was in CM containing newborn serum, whereas the experiments

described here were in BRL-medium containing foetal serum. Evidence has previously been presented that serum may have complex effects on cell differentiation (Section 4.5.4. and 4.6.). This issue should be resolved by observation of the effects of HMBA in BRL-medium containing newborn calf serum.

BRL-medium has also been found to reduce the toxic effects of retinoic acid towards PSA4-derived EC cells (Sections 3.2.2. and 4.5.3.). This is most evident for cultures growing in the presence of foetal serum where the $L.D._{50}$ is increased from approximately $10^{-9}M$ in CM to greater than $10^{-5}M$ in BRL-medium (Table 3.3, Fig.3.2). This action is clearly not due to inactivation of the retinoid since differentiation of PSA4TG12 is induced by $10^{-9}M$ retinoic acid in BRL-medium containing foetal serum (Section 3.2.1., 3.1). High toxicity to PSA4-derived cells in CM containing foetal serum could arise because the type of spontaneously differentiated cell obtained in this medium is highly susceptible to retinoic acid. This could be investigated by inducing cultures with retinoic acid for only 24 hours, or by allowing the cells to differentiate spontaneously in CM, then treating with retinoid. In BRL-medium containing foetal serum and supplemented with retinoic acid a different type of differentiated cell is obtained (see above) which is clearly resistant to retinoid toxicity.

Retinoic acid is less toxic in CM containing newborn serum, so the effect of BRL-medium is less pronounced (Section 4.5.3.). It does enhance the survival of the differentiation-defective clone PT2md1, however (Fig.4.3b). As BRL-medium has been shown to reduce the differentiation of PC13.5 cells induced by retinoic acid (Section 5.3.5.), this supports the hypothesis that mortality of PT2md1 is associated with the induction of commitment (Sections 4.5.4. and 4.6.).

5.4. DISCUSSION

The evidence presented in this Chapter demonstrates that medium conditioned by BRL cells inhibits the differentiation of EC cells induced by three different stimuli; plating in the absence of feeders, aggregation, and exposure to retinoic acid. The effect on spontaneous differentiation in monolayer culture is much greater than that previously reported for medium conditioned by STO feeder

layers (Smith and Hooper, 1983) or by a variety of dividing cell types (Koopman and Cotton, 1984). It is possible that this may reflect a qualitative rather than just a quantitative distinction, but this awaits purification of the activities present in the different media (see following Chapter). The failure of medium conditioned by PC13.5 EC cells to cause any inhibition of spontaneous differentiation of PT2 indicates either that PSA4-derived lines do not respond to the differentiation-inhibiting properties of ECDGF, that its effective concentration in conditioned medium is below the response threshold of PSA4, or that its effects are overcome by other activities in the conditioned medium. These possibilities can only be investigated using purified ECDGF. It should be noted, however, that this factor is unstable (Heath and Isacke, 1984) and that its inhibitory action on OC15S2 differentiation is very inefficient (J.Heath, personal communication).

The action of BRL-medium has been shown to entail no significant cell selection and to be fully reversible. It enables for the first time feeder-free culture of homogeneous stem cell populations of feeder-dependent EC cells. This may represent a considerable advance as feeder-dependent cells generally have a greater developmental potential *in vivo* than their feeder-independent counterparts (Martin and Evans, 1975a,b; Hogan, 1976; Nicolas *et al*, 1981a). The availability of pure populations of these cells should facilitate developmental, biochemical, genetic and molecular studies.

The extension of the action of BRL-conditioned medium to the EK line B2B2 may prove to be of even greater significance, not only for the type of study mentioned above, but also because it may enable the isolation of EK lines in the absence of feeders. This is of some importance as all existing EK lines have been derived on feeder layers and there is a theoretical possibility that transfer of genetic material from the feeders to the inner cell mass cells occurs and causes transformation of the latter.

The fact that BRL-conditioned medium is effective on EK cells further suggests that this activity may play a major role in normal embryonic development. Therefore a major effort should be directed at its purification and characterisation (see following Chapter). If the activity can be purified it could be of considerable value in embryo culture, both for experimental purposes and for *in vitro* fertilization (IVF) programmes. The observations on endodermal outgrowths from embryoid bodies (Section 5.3.4.) and retinoic acid-induced

parietal endoderm-like cells (Section 5.3.6.) suggest that BRL-medium may support the growth of extraembryonic endoderm in addition to any effects it has on inner cell mass cells. This is substantiated by the finding that BRL-medium enables feeder-free culture of a feeder-dependent embryo-derived cell line, Mod-1, which exhibits many of the characteristics of parietal endoderm (Smith, 1985). It will be of interest to determine whether this activity is the same as, or distinct from, that which inhibits EK and EC cell differentiation.

The effects of BRL-conditioned medium on EC cell differentiation in aggregates (Section 5.3.4.) are rather surprising. It does not completely prevent differentiation. On the contrary, an extensive outer layer of endoderm develops quite rapidly and encases the inner core of EC cells. Development is arrested at this stage, however. The finding that both cavitation and differentiation of the EC core are blocked supports the proposal that these two processes are intimately associated (Martin *et al*, 1977; Uno, 1982). Much of what is known about the development of embryoid bodies is based on comparisons between cavitating and non-cavitating EC lines (Martin *et al*, 1977; Boyd *et al*, 1984). The effect of BRL-conditioned medium provides a new permutation in the investigation of the control of cavitation and further differentiation in embryoid bodies and, by analogy, in the egg cylinder.

Three issues come immediately to mind. Firstly, it should be determined whether the blocked development of EC cores arises from specific inhibition of a developmental pathway, or merely represents a delay in normal developmental processes. The latter could result from enhanced viability and proliferation of the EC cells retarding the onset of necrosis and cavity formation, for example. This can be tested by maintaining embryoid bodies in suspension culture for longer than the 6 day maximum used in this study. If the effect is a genuine inhibitory one, the question arises as to whether or not it is reversible *in situ*. (It has already been shown to be reversible if the embryoid bodies are allowed to attach to a substratum, Section 5.3.4.) This could be addressed by initiating suspension cultures in 60% BRL-conditioned medium, then transferring them to normal EfC10 for a period before histological analysis.

A second major question is whether or not visceral endoderm is produced in BRL-conditioned medium as it has been proposed that this tissue may control

cavity formation (Martin *et al*, 1977). A tentative indication that visceral endoderm may be present in some of the simple embryoid bodies generated in BRL-conditioned medium is provided by the morphology of parts of the endoderm layer (Plate 5.8E). This requires confirmation by ultrastructural analysis and immunological studies. If substantiated it would indicate that BRL-conditioned medium may act directly on the EC core to prevent further differentiation, though suppression of an inductive stimulus from the visceral endoderm could not be excluded.

Thirdly, in view of its widespread effects on EC cell differentiation and the finding that it overcomes the effect of BRL-medium on monolayer cultures (Sections 5.3.5. and 5.3.6.), it would be of interest to investigate whether retinoic acid can overcome the block imposed by BRL-conditioned medium and induce cavity formation and/or further differentiation.

Evidence consistent with a direct effect of BRL-conditioned medium on the EC cores is provided by the outgrowths which develop when the simple embryoid bodies are allowed to reattach to the substratum (Section 5.3.4.). When attachment is in normal CM the differentiated outgrowths contain a variety of tissue and cell types (Plate 5.9). The EC line S2, which only forms simple embryoid bodies due to some genetic or epigenetic block (Martin and Evans, 1975a,b), likewise differentiates on attachment (Evans and Martin, 1975; Martin and Evans, 1975c). When reattachment of arrested PSA4 embryoid bodies is in BRL-medium, however, this differentiation is inhibited and the cells which grow out resemble extraembryonic endoderm (Plate 5.10), though morphological evidence alone cannot preclude the possible presence of primitive bone- or cartilage-forming cells.

BRL-medium has also been shown to inhibit the induction of differentiation of feeder-independent PC13.5 EC cells by retinoic acid (Section 5.3.5.). Unlike the inhibition of spontaneous differentiation of monolayers of feeder-dependent cells, however, this effect is only partial. This presumably reflects the greater potency of retinoic acid as an inducer of differentiation (see Section 4.5.4.) The same explanation probably underlies the capacity of retinoic acid to overcome the inhibition of PSA4 and B2B2 differentiation imposed by BRL-medium (Section 4.5.6.). At first sight these two observations appear contradictory. However, the observation that retinoic acid induces differentiation in BRL-medium is in fact the same for PC13.5, PSA4 and B2B2. The partial

inhibition manifest with PC13.5 may also apply to the feeder-dependent lines. Unfortunately, this cannot be directly assessed because these cells differentiate spontaneously in CM and are highly susceptible to retinoic acid toxicity (Section 3.2.2. and 4.5.3.).

The finding that retinoic acid induces differentiation of PSA4 and B2B2 in BRL-medium is interesting in several respects. Firstly, it demonstrates that the inductive potential of retinoic acid is not confined to feeder-independent EC cells, but operates on feeder-dependent EC and EK cells. The latter observation indicates that retinoid-induced differentiation is not merely an artefact of transformation (but see comment above *re* the possibility that EK cells could be transformed) or *in vitro* selection. Secondly, it shows that inhibition of commitment in monolayer cultures imposed by BRL-medium can be circumvented, but that as with aggregation, differentiation appears confined to a particular cell lineage, or even to a specific cell type. In both cases it appears that only extraembryonic endoderm cells develop. In monolayer culture this may be because retinoic acid specifically induces differentiation of PSA4 and B2B2 into primitive and/or parietal endoderm and not into other cell types. Alternatively, the direction of differentiation may be determined by BRL-medium. Monolayers of F9 EC cells differentiate almost exclusively into primitive and parietal endoderm when exposed to retinoic acid in monolayer culture (Strickland *et al*, 1980; Hogan *et al*, 1981). However, other EC cells differentiate into different cell types, depending on both the particular EC line and the culture conditions (Jetten *et al*, 1979; Edwards and McBurney, 1983). In general retinoic acid appears to stimulate differentiation of the same form as that which occurs spontaneously in the EC line in question. This suggests that retinoic acid acts to trigger EC cell commitment but does not directly determine the ensuing differentiation program, which is controlled by genetic and environmental factors (see Section 1.5. for further discussion). The normal differentiation response of PSA4 to retinoic acid is uncertain, as the retinoid is highly toxic to these cells in CM containing foetal serum (Section 3.2.2.). It might be possible to circumvent this problem by exposing the cells to retinoid for only 24 hours, or by performing the experiments in CM containing newborn serum, in which the retinoid is less toxic (Section 4.5.3.). Such experiments would be complicated by the high level of spontaneous differentiation in CM, however.

Even if retinoic acid was found to specifically direct differentiation of PSA4 and

B2B2 into parietal endoderm, it would still be consistent with the intriguing possibility that cell determination in BRL-medium is restricted to the extraembryonic endoderm lineage. Differentiation solely into extraembryonic endoderm could arise because BRL-medium promotes commitment to this developmental pathway, or because it suppresses other forms of commitment. The latter hypothesis more readily explains the observations on blocked differentiation of the EC cores of embryoid bodies discussed above. Moreover, the fact that BRL-medium completely inhibits spontaneous commitment in monolayer cultures implies that the former hypothesis is only valid if BRL-medium has two separate effects; inhibition of the initiation of any commitment, and promotion of commitment, once initiated, to the extraembryonic endoderm lineage.

It is possible that the differentiation of EC and EK cells into extraembryonic endoderm is an aberrant behaviour (see Section 1.4.), which has escaped from the control of the normal regulatory systems operative in the embryo. However, normal embryonic development does provide a basis for a lineage-specific control of commitment of the type proposed above since the primitive endoderm differentiates from the ICM prior to implantation, whilst proamniotic cavity formation and further differentiation only occur after implantation of the blastocyst into the uterine wall (see Section 1.2.2.). Furthermore, differentiation of primitive endoderm is accompanied by preferential inactivation of the paternal X chromosome (Takagi and Sasaki, 1975; West at al. 1977;), whereas X-inactivation is random in derivatives of primitive ectoderm. Consequently it might be expected that determination to form extraembryonic endoderm would be separately regulated from commitment to other lineages.

The finding that undiluted BRL-conditioned medium appears to arrest growth of EC cells (Section 5.2.) may also have a basis in early embryonic development, since inner cell mass cells do not proliferate continuously but cease division prior to implantation (Handyside and Hunter, 1984). Certainly this phenomenon is of interest, since the division rate of undifferentiated EC cells generally appears impervious to environmental influences, that is the cells die rather than become quiescent (Heath, 1983). Firstly, it must be confirmed by cell counts and measurements of DNA synthesis that this medium does inhibit cell division. Secondly, it should be determined whether growth arrest is merely a consequence of depletion of nutrients from the medium, or results from the differentiation-inhibiting or some other activity produced by the BRL cells. If

growth is arrested, the failure of the EC cells to differentiate when growth resumes (Section 5.2.) demonstrates that inhibition of cell division is not a sufficient condition for induction of differentiation. Such a system could also be used to investigate the requirement of retinoic acid-induced commitment for active cell division. Another interesting question concerns expression of the proto-oncogene c-*myc*. This is associated with cell division (Robertson, 1984; Kelly *et al*, 1984) and is expressed in high levels by EC cells (Campisi *et al*, 1984). Its expression falls during retinoic acid-induced differentiation, however (Campisi *et al*, 1984). The related N-*myc* gene is also actively expressed by EC cells and its expression too falls on differentiation into extraembryonic endoderm, induced either by retinoic acid or by aggregation (Jakobovits et al, 1985). Reduced expression of N-*myc* has been reported to precede cell cycle changes and morphological differentiation of neuroblastoma cells exposed to retinoic acid (Thiele *et al*, 1985). If decreased expression of these genes is associated with EC cell commitment, they may continue to be expressed in PSA4 cells in undiluted BRL-medium, even though the cells are not dividing.

The action of retinoic acid on feeder-dependent cells cultured in BRL-medium also supplies a system for directing the differentiation of EC and EK cells along different developmental pathways in monolayer culture. The only previous observation of alternative forms of differentiation in monolayers followed transfection of F9 EC cells with the c-*fos* oncogene (Muller and Wagner, 1984). The transfected cells differentiated at high frequency into a cell type distinct from the parietal endoderm cells induced by retinoic acid plus dibutyryl cyclic-AMP. Other reports of bidirectional differentiation *in vitro* have depended on differences, either between monolayers and aggregates (Speers *et al*, 1979; Hogan *et al*, 1981; Jones-Villeneuve *et al*, 1982) or between aggregates under different conditions (Edwards and McBurney, 1983). Whilst aggregation permits the study of intercellular interactions, it incurs the disadvantage that mixtures of at least two (EC and differentiated), and usually more, cell types are generated, rendering difficult any molecular analyses of commitment and differentiation. The latter are facilitated by homogeneous differentiation into discrete cell types (see for example, Wang *et al*, 1984; Sejerson *et al*, 1985). Retinoic acid-induced differentiation of PSA4 or B2B2 yields a cell type quite distinct from the major type(s) generated by spontaneous differentiation in CM containing foetal serum (yet a third type of cell may be produced in CM

containing newborn serum, as discussed in Section 4.5.4.). The former show many resemblances to parietal endoderm and, as they can be obtained in large numbers, it should be relatively straightforward to confirm this identification. The latter may be a mixture of cell types or could be composed of alternative morphological configurations of a single cell type dependent on local cell density. This requires clarification by screening with a variety of antisera directed against a battery of cell-specific antigens. If the cells should turn out to be of a single type, or at least of a single lineage, comparison with retinoic acid-induced differentiation in BRL-medium would provide an ideal system for analysing the molecular events underlying cell determination. This has long been one of the principal aims of investigators working on teratocarcinoma cells (Martin, 1975).

CHAPTER 6

PARTIAL PURIFICATION OF DIFFERENTIATION INHIBITING ACTIVITY FROM MEDIUM CONDITIONED BY BUFFALO RAT LIVER (BRL) CELLS

6.1. INTRODUCTION

The findings reported in the previous Chapter demonstrate that medium conditioned by Buffalo rat liver (BRL) cells is a potent inhibitor of EC cell differentiation. As discussed in Section 5.4., the source of this inhibitory action may be of considerable theoretical, experimental and biomedical interest.

The original rationale for examining the effect of BRL-conditioned medium on EC cells (Section 5.2.) was that these cells produce large amounts of insulin-like growth factor-II (IGF-II), previously known as multiplication-stimulating activity (MSA) (Dulak and Temin, 1973a,b). This is a member of the somatomedin family of growth factors which promotes DNA synthesis and multiplication of a variety of foetal and adult cell types (Rechler *et al*, 1981; Herrington *et al*, 1983). It is one of the few growth factors for which undifferentiated EC cells possess functional receptors (Nagarajan *et al*, 1982; Heath, 1983). Physiological concentrations of IGF-II support proliferation of F9 and PC13 EC cells in serum-free medium (Nagarajan *et al*, 1982; Heath and Deller, 1983). Additional evidence that it may be an important embryonic growth factor comes from its higher concentration in foetal than in adult serum (Moses *et al*, 1980a) and its production by a variety of embryonic and neonatal cell types (Dulak and Temin, 1973a; Anderson *et al*, 1983b; Adams *et al*, 1983).

Mature rat IGF-II is a single chain polypeptide of 67 residues with molecular weight of 7,484 (Marquardt *et al*, 1981). A range of larger precursors also show biological activity (Moses *et al*, 1980b,c). The largest of these is pro-IGF-II with a molecular weight of 17,619 (Whitfield *et al*, 1984). BRL cells also produce a somatomedin binding protein and in conditioned medium IGF-II is mostly associated with this carrier protein in a complex of molecular weight 50-60,000 (Knauer and Smith, 1981). This complex dissociates at acid pH.

Medium conditioned by STO feeders also inhibits EC cell differentiation (Smith and Hooper, 1983). It is not known whether STO cells produce IGF-II, but this factor is synthesized by other embryonic fibroblasts (Adams *et al*, 1983).

Koopman and Cotton (1984) have reported that the activity in feeder-conditioned medium resides in a polypeptide of apparent molecular weight 57,000 on neutral gel filtration. They have named this protein Differentiation Retarding Factor (DRF). Unfortunately these workers did not investigate the mitogenic potential of DRF, or examine the effects of acid treatment. Therefore, it cannot be precluded that DRF consists of the intact IGF-II-carrier complex. Alternatively, it may represent a distinct protein species, which may or may not be produced by BRL cells. The report (Koopman and Cotton, 1984) that feeder-conditioned medium retards rather than inhibits differentiation suggests that feeders and BRL cells may produce qualitatively different activities (see also Section 5.4.). The activity present in BRL-conditioned medium has been named "Differentiation Inhibiting Activity" or "DIA", reflecting the possibility that it may be distinct from DRF. The results of efforts to purify DIA and clarify its relationship to IGF-II are reported in Section 6.2.

In parallel with the attempt at direct purification of DIA, several factors considered as possible candidates for this activity were screened for their effects on feeder-free monolayer cultures of PSA4. Media conditioned by various cell lines were likewise assayed. The results of these studies are presented in Section 6.3.

6.2. PARTIAL PURIFICATION OF DIFFERENTIATION INHIBITING ACTIVITY

6.2.1. Introduction

Initial stages in the attempt to purify DIA from BRL-conditioned medium followed the purification scheme of Marquardt *et al* (1981) for IGF-II (Section 2.16.).

DIA activity was monitored on feeder-free cultures of PSA4 (Section 2.10.2.). Differentiated cultures can be unequivocally distinguished from EC cultures by their morphology after 4 days. A semi-quantitative assay procedure was established in 16mm Linbro wells (Section 2.10.2.). A unit of DIA was defined as that activity in 1ml of CM which permitted differentiation equivalent to that obtained in 1ml of 50% (v/v) BRL-conditioned medium. Under such conditions the cultures are predominantly embryonal carcinoma but a significant

subpopulation (5-10%) of differentiated cells is also present (see Table 5.2). The activity present in various fractions was estimated by titration down to this endpoint.

Mitogenic activity was monitored by assaying the stimulation of [^{3}H]-thymidine incorporation by quiescent NRK 49F rat kidney fibroblasts (Section 2.17.).

6.2.2. Production of Concentrated Conditioned Medium and Dialysis Against Acetic Acid

Serum-free conditioned medium was collected from a confluent microcarrier culture of BRL cells as detailed in Section 2.16. A total volume of 2.4l of medium was concentrated 10-12-fold using four immersible-CX ultrafilters set up in parallel. When diluted with CM this concentrate supported the feeder-free proliferation of undifferentiated PSA4 EC cells.

The concentrated conditioned medium was dialysed exhaustively against 0.1M acetic acid. During dialysis there was some precipitation of material. The precipitate was removed by high speed centrifugation. The clear supernatant so obtained was lyophilized and reconstituted in 6ml of 0.67M acetic acid. This fraction was termed the "Dialysate". An aliquot (1ml) was retained for characterisation and the remainder was utilised for further purification steps (following Section).

The Dialysate, when neutralized with 1M NaOH, diluted with serum-free medium and added to CM, did inhibit the spontaneous differentiation of PSA4 in the absence of feeders. However, the EC cells grew as tight clumps under these conditions and aggregates frequently detached from the substratum. Subsequently it was found that monolayer growth comparable to that in BRL-medium was obtained from preparations of Dialysate lyophilized to dryness, reconstituted in a small volume of 0.1M acetic acid and diluted 1:19 with MSA buffer (Section 2.16). Preparations containing the equivalent of 2µl of Dialysate possessed 1 unit of Differentiation Inhibiting Activity as defined in the previous Section. This represents a 250-fold concentration relative to neat BRL-conditioned medium and a recovery of approximately 62.5% (Table 6.1).

The Dialysate was also mitogenic for NRK 49F rat fibroblasts as measured by stimulation of [^{3}H]-thymidine incorporation (Table 6.2).

Table 6.1
Purification of Differentiation Inhibiting Activity

Fraction	Protein (mg)	DIA Units	Sp. Act. (Units/mg)	Yield	Purification
Medium	60.00*	4800	80.0	100.0	1.00
Dialysate	36.72	3000	81.7	62.5	1.02
P-10	27.50	2880	104.7	60.0	1.31
G-75	3.24	1620	500.0	33.8	6.25

*Determined after removal of low molecular weight material by gel filtration on a Sephadex G-25M Column
^All figures corrected to allow for the proportion of fractions utilized in characterisation assays

Table 6.2
Stimulation of [^{3}H]-Thymidine Incorporation by NRK 49F Fibroblasts by Fractions Containing DIA

Fraction	Dilution Factor*	Specific c.p.m.^ Expt.I	Expt.II
Foetal calf serum	10	260,659	261,473
Conditioned-medium	10	591	4,806
Dialysate	100	107,522	-
P-10	50	253,542	-
"	100	220,940	-
"	250	-	127,531
P-10,boiled	100	117,114	-
G-75	70	-	156,145

*Final dilution in incubation volume of 1.0ml
^C.p.m. incorporated in the presence of test sample minus c.p.m. incorporated in responseto MSA buffer alone.
Data are the means of duplicate determinations performed as detailed in Section 2.17. in two separate experiments on fractions from a single purification.

6.2.3. Gel Filtration Chromatography on Bio-Gel P-10

Bio-Gel P-10 is a chromatography matrix used to fractionate molecules with a molecular weight less than 15,000. A 2.5x80cm^2 column equilibrated with 0.67M acetic acid was calibrated with the following molecular weight standards; bovine haemoglobin (68,000), horse myoglobin (17,000), cytochrome C (12,400), bovine insulin (5,700) and bacitracin (1,400). Haemoglobin and myoglobin eluted in the excluded volume (V_o). The elution volumes of the other marker proteins are shown in Fig.6.1.

The reconstituted Dialysate (5ml) was applied to this column and eluted in 0.67M acetic acid at a flow rate of 13ml/h. The bulk of the protein, monitored

by absorption at 280nm, eluted in the void volume. Fractions (6.5ml) were assayed for DIA and mitogenic activity as described (Sections 2.10.2., 2.16. and 2.17.). The results are depicted in Fig.6.1.

It is apparent that mitogenic activity retarded by the column eluted at the expected positions (Marquardt *et al*, 1981) for mature rat IGF-II (apparent molecular weight 6,500-7,000) and slightly larger precursors. The major mitogenic peak is present in the void volume, however.

Only fractions in the excluded volume showed any DIA activity on PSA4 cultures at a final dilution of 1:24. Five fractions (total volume 32.5ml) exhibiting such activity were pooled, lyophilized and reconstituted in 6ml of 0.67M acetic acid. Table 6.1 shows that this "P-10 Fraction" contains 80% of the activity present in the Dialysate. An aliquot (1.0ml) was retained for characterisation and the remainder was applied to a G-75 column (following Section).

Some growth factors are resistant to heat-denaturation (DeLarco and Todaro, 1978b; Antoniades *et al*, 1979; Rizzino *et al*, 1983). Consequently it was decided to investigate the thermal stability of both differentiation inhibiting and mitogenic activities in extracts of BRL-conditioned medium. Initial observations on heat-treated preparations of serum-free conditioned medium indicated that incubation at temperatures up to 80°C for 10 minutes had no significant effect on inhibition of differentiation in PSA4 mass cultures. In boiled samples there was considerable cell death (presumably arising from inactivation of essential constituents in the medium or production of toxic substances), but the majority of surviving cells proliferated without differentiating. As mitogenic activity in untreated BRL-conditioned medium is very low (Table 6.2), meaningful data on the heat-stability of this activity could not be obtained in these preparations. Therefore further characterisation was carried out on the P-10 Fraction.

The data presented in Table 6.3 demonstrate that DIA is reduced by between 50% and 60% following incubation at 100°C for 10 minutes, though significant activity persists through this treatment. The results in Table 6.2 indicate that mitogenic activity in the P-10 Fraction likewise decreases by around 50% on boiling.

Fig.6.1 Bio-Gel P-10 Gel Filtration of BRL-Conditioned Medium

BRL "Dialysate" (5ml) was applied to a P-10 column and eluted as described in Section 2.16. Aliquots of each fraction were assayed for mitogenic activity on NRK 49F rat fibroblasts (Section 2.17.), indicated by the blue line, and for the capacity to inhibit the spontaneous differentiation of PSA4 plated on gelatin (Section 2.10.2.), indicated by the black bar (DIA).

The void volume, V_o, was determined using bovine haemoglobin. Elution positions of the standards used to construct a linear plot of log molecular weight versus V_e/V_o are marked:-

	Molecular Weight
C: cytochrome C	12,400
I: insulin	5,700
B: bacitracin	1,400

Fig.6.1 Bio-Gel P-10 Gel Filtration of BRL-Conditioned Medium

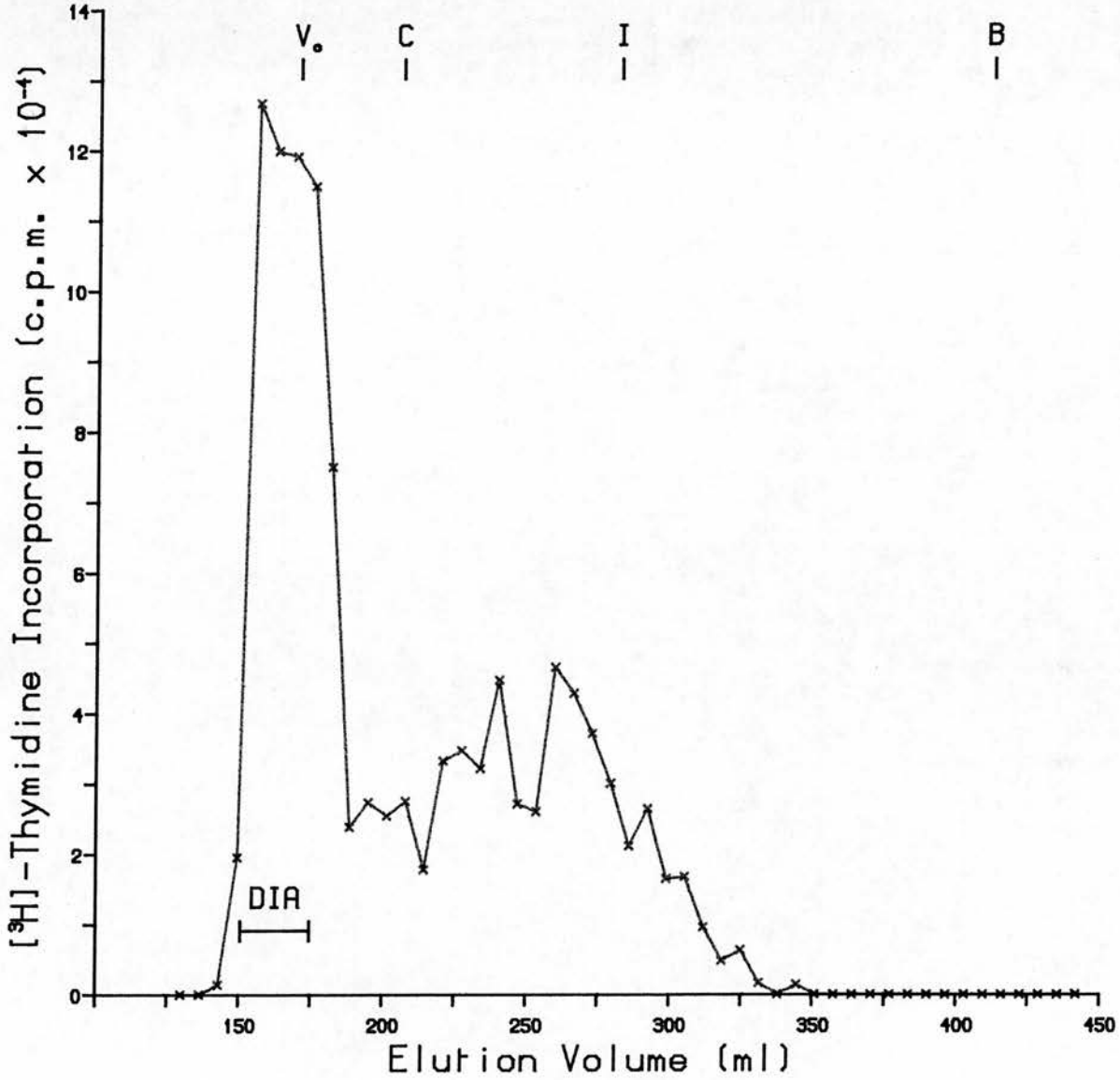


Table 6.3
The Effect of Heating to 100°C on DIA Activity

P-10 Sample	DIA Units/ml
Before boiling	400
After boiling	160

An aliquot (0.2ml) of P-10 Fraction, neutralized with 5M NaOH was made up to 1.0ml with MSA buffer and divided into two 0.5ml samples. One sample was placed in a boiling water bath for 10 minutes. Both samples were then assayed for DIA activity on PSA4 cultures as described in Section 2.10.2.

6.2.4. Gel Filtration Chromatography on Sephadex G-75

As DIA does not co-elute with mature IGF-II but is excluded from a P-10 column (previous Section), a second gel filtration step was introduced. Sephadex G-75 is a widely used gel chromatography matrix suitable for fractionation of proteins of intermediate size (15-70,000 Daltons). A 2.5x90cm^2 column was prepared and equilibrated with 0.67M acetic acid. A standard plot of log molecular weight versus the ratio of elution volume to void volume (V_e/V_o) was constructed using blue dextran (2,000,000), bovine serum albumin (66,000), carbonic anhydrase (29,000) and cytochrome C (12,400) as molecular weight markers. The reconstituted P-10 Fraction (5ml) was applied to this column and eluted in 0.67M acetic acid at a flow rate of 12ml/h. Each fraction (3ml) was assayed for DIA activity on PSA4, and alternate fractions were assayed for the ability to stimulate DNA synthesis by NRK 49F cells. The elution profiles of protein (A_{280}), DIA and mitogenic activity are shown in Fig.6.2.

Most of the protein again eluted in the void volume. This exhibited no biological activity in either the DIA or mitogen assays. Three regions of mitogenic activity were present in the eluate. The first (I) eluted immediately after the excluded volume with an apparent molecular weight of 60,000 and displays the lowest activity. The second (II) and third (III) peaks eluted close to one another in the 15-40,000 molecular weight range. A similar profile was obtained on repetition of the mitogenic assay in this region of the effluent, confirming that there are two peaks, with peak III having a greater specific activity than peak II. DIA activity was present in 9 fractions (total volume 27ml) of the effluent between, and overlapping, peaks II and III of mitogenic activity

Fig.6.2 Sephadex G-75 Filtration of BRL-Conditioned Medium

"P-10 Fraction" (5ml) was applied to a G-75 column and eluted as described in Section 2.16. Protein elution (A_{280}) is shown by the red line. Alternate fractions were assayed for mitogenic activity on NRK 49F rat fibroblasts (Section 2.17.), indicated by the blue line, and all fractions were assayed for the capacity to inhibit the spontaneous differentiation of PSA4 plated on gelatin (Section 2.10.2.), indicated by the black bar (DIA).

The void volume, V_o, was determined with blue dextran. Elution positions of the standards used to construct a linear plot of log molecular weight versus V_e/V_o are marked:-

	Molecular Weight
A: albumin, bovine	66,000
CA: carbonic anhydrase	29,000
C: cytochrome C	12,400

Fig.6.2 Sephadex G-75 Gel Filtration of BRL-Conditioned Medium

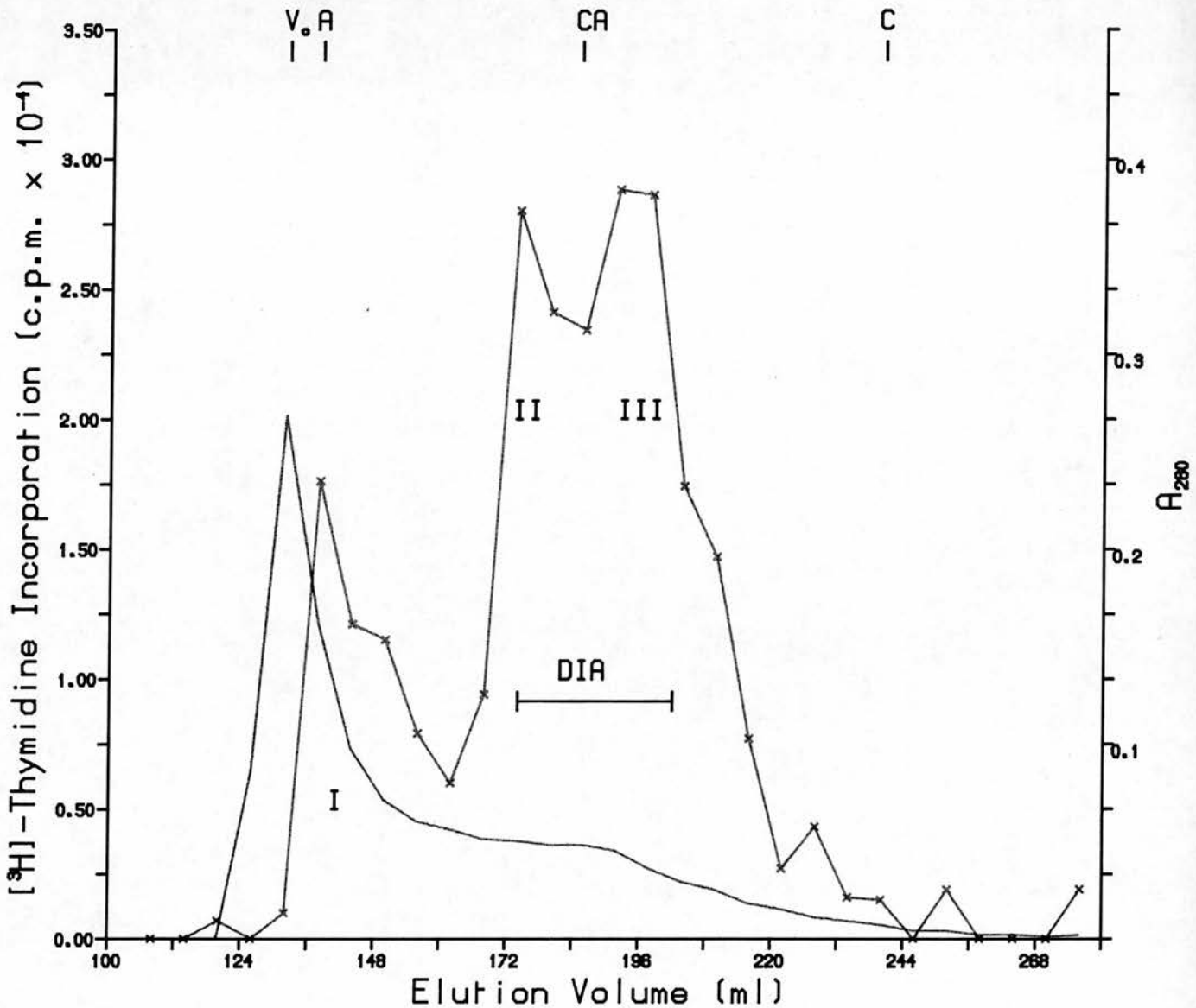
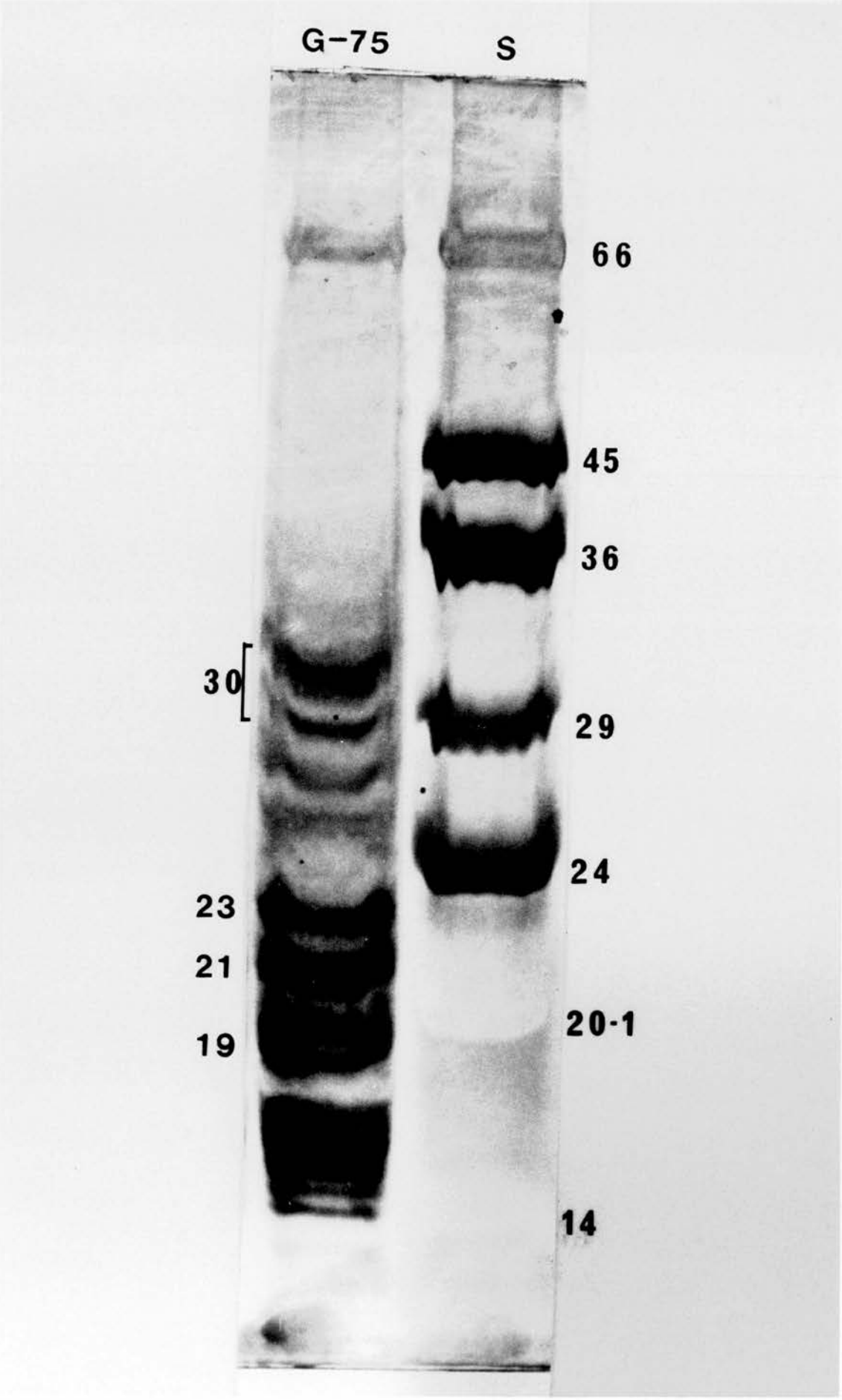


Plate 6.1 Gel Electrophoresis Profile of "G-75 Fraction"

SDS-PAGE was carried out as described in Section 2.18. The gel was initially stained with kenacid blue, then destained and stained with silver (Section 2.18). The track labelled **S** contains the Sigma SDS-70L molecular weight standards:-

	Molecular Weight (kD)
Albumin, bovine	66
Ovalbumin	45
Glyceraldehyde-3-phosphate dehydrogenase	36
Carbonic anhydrase	29
Trypsinogen	24
Trypsin inhibitor	20.1
α-lactalbumin	14.2

The track marked **G-75** was loaded with test sample under reducing conditions (Section 2.18.). The labelled bands are those revealed by staining with kenacid blue alone. The doublet at 30kD appears as a single broad band when stained with kenacid blue and under non-reducing conditions. The band at 23kD is absent under non-reducing conditions.


G-75
S
66
45
36
30
29
24
23
21
20-1
19
14

(apparent molecular weight 20-35,000). No inhibition of PSA4 differentiation was detected in response to any other fractions (diluted 1:24).

The volume remaining in the DIA-containing fractions after these assays and SDS-PAGE analyses (following Section) was pooled, lyophilized and reconstituted in 0.1M acetic acid (one tenth of the original volume). This "G-75 Fraction" is both highly mitogenic (Table 6.2) and a potent inhibitor of PSA4 differentiation (Table 6.1). It represents a six-fold purification of DIA relative to conditioned medium and a recovery of 33.8%. Aliquots of this preparation were subjected to SDS-PAGE electrophoresis (following Section).

6.2.5. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous vertical gel electrophoresis was performed as detailed in Section 2.19. on alternate fractions of G-75 eluant containing DIA. Three major bands stained with kenacid blue in all non-reduced samples at positions corresponding to apparent molecular weights of 30,000, 21,000 and 19,000. Reduced samples generated an additional band with an apparent molecular weight of 23,000 and in some cases the broad band at around 30,000 was resolved into a doublet. This banding pattern was reproduced by the pooled G-75 Fraction (Plate 6.1).

The silver-staining technique (Oakley *et al*, 1980) enables detection of as little as 10ng of protein in polyacrylamide gels, though it does not stain all proteins and is not quantitative. The silver stain disclosed additional proteins in all DIA samples, notably at apparent molecular weights of 66,000 and 15-16,000 (Plate 6.1). A band at 35,000 was resolved only in the penultimate DIA-containing fraction and in the column fraction which eluted immediately after DIA activity. Otherwise protein composition appeared relatively constant in all DIA-containing samples.

6.2.6. Summary

The results reported in this Section demonstrate that DIA is a non-dialyzable, acid-stable and relatively heat-resistant activity. It is apparently quite distinct from mature IGF-II, as indicated by its exclusion from Bio-Gel P-10 (Fig.6.1). DIA elutes with an apparent molecular weight of 20-35,000 on Sephadex G-75

chromatography.

Three mitogenic peaks are also resolved by G-75 gel filtration. Peak I, apparent molecular weight 60,000, is well separated from DIA. It is too large for any known growth factor and may be an artefact arising from incomplete dissociation of IGF-II-carrier complexes, or the persistence of aggregates of IGF-II molecules and/or non-specific binding of IGF-II to other proteins such as albumin. This has not been reported by most other workers following acid-treatment (Moses *et al*, 1980b; Marquardt *et al*, 1981; J.Heath, personal communication). It may arise from the slightly milder conditions, 0.67M rather than 1.0M acetic acid, used here (necessitated by acid-sensitive components of the chromatography column).

The above explanation may also account for peak II, apparent molecular weight 35.000. Alternatively this could represent a large growth factor. It has recently been shown that BRL cells produce transforming growth factor-β (TGF-β), molecular weight 25,000 (Massague *et al*, 1985). This may not be present in the P-10 Fraction applied to the G-75 column, however, as it is anomalously retarded by Bio-Gel chromatography matrices (Assoian *et al*, 1983; Massague *et al*, 1985). It remains possible that BRL cells may produce other large mitogens. Platelet-derived growth factor, for example, has a molecular weight of 30,000 (Heldin *et al*, 1979). Florini *et al*, (1984), have reported a growth-promoting activity in acid-treated extracts of BRL-conditioned medium with an apparent molecular weight of 31,000.

Peak III probably consists, at least in part, of the pro-IGF-II precursor, molecular weight 17,619, which is present in abundance in BRL-conditioned medium (Moses *et al*, 1980b; J. Heath, personal communication) and was apparently excluded from the P-10 column in this preparation (Section 6.2.3.). It may also contain other species, as proposed for peak II. It is further possible that peaks II and III may be composed largely of a single activity, with their separation resulting from some artefact of the column chromatography or from the action of an inhibitor. An antagonist of TGF-β has been characterised, for example, which splits the elution profile into a double peak of bioactivity (Frolik *et al*, 1984; Massague *et al*, 1985).

The elution position of DIA between and overlapping two peaks of mitogenic activity can be interpreted in at least three different ways:-

1. It may be a unique species, distinct from any mitogen.
2. It may be a single growth factor, perhaps present in multiple forms, with the separation of peaks II and III being artefactual, as discussed above.
3. It may arise from the concerted action of two mitogens, i.e. the combination of peaks II and III.

SDS-PAGE analysis of DIA-containing fractions does not aid in distinguishing between these alternatives. Protein composition appears relatively constant in all the fractions analysed. Three major proteins are present in non-reduced samples and silver-staining reveals several other species. Only the broad kenacid blue-staining band with an apparent molecular weight of 30,000 can tentatively be assigned to a known protein. It probably represents the low molecular weight IGF-II carrier protein which is produced in very large amounts by BRL cells and is present in acid-treated fractions (Knauer and Smith, 1980; J. Heath, personal communication). Under non-reducing conditions this migrates as a single band in SDS polyacrylamide gels, with an apparent molecular weight of 31,500, whilst in the presence of 2-mercaptoethanol two adjacent bands are generated in the 30-32,000 molecular weight range. The identity of the remainder of the proteins in the G-75 Fraction and which, if any, of them corresponds to DIA remain unknown. Neither PDGF (molecular weight 30,000 under non-reducing conditions, 14-17,500 in the presence of 2-mercaptoethanol) nor TGF-β (molecular weight 23-25,000 non-reduced, 12,500 reduced), both of which are disulphide cross-linked dimers are present in amounts detectable by kenacid blue-staining. These growth factors could be present in the electrophoresis samples in only nanogram quantities and still elicit biological responses, however, as they are active at very low concentrations (10^{-11}-10^{-9}M). It is possible that bands corresponding to non-reduced and reduced PDGF (or some PDGF-like factor) and TGF-β are amongst those resolved by silver-staining (Plate 6.1).

6.3. THE EFFECTS OF PURIFIED GROWTH FACTORS AND OF VARIOUS CONDITIONED MEDIA ON DIFFERENTIATION OF PSA4 EMBRYONAL CARCINOMA CELLS

6.3.1. Purified Growth Factors

In addition to the attempt to purify DIA reported in the previous Section, several growth factors were obtained in pure form and their effects on feeder-free cultures of PSA4 were examined directly. The factors tested were bovine insulin (tissue culture grade), rat insulin-like growth factor-II (IGF-II), a commercial preparation of rat multiplication-stimulating activity (MSA), porcine platelet-derived growth factor (PDGF), murine epidermal growth factor (EGF) and human transforming growth factor-β (TGF-β).

Insulin displays growth-promoting activity in addition to its well-known metabolic effects (King and Kahn, 1981). At superphysiological concentrations (1μg/ml) it supports the serum-free growth of F9 and PC13 EC cells (Rizzino and Crowley, 1980; Heath and Deller, 1983), though how this action is mediated remains uncertain as PC13 does not possess insulin receptors (Heath *et al*, 1981) and insulin does not cross-react with the IGF-II receptor (Massague and Czech, 1982; Heath, 1983).

Nanomolar concentrations of insulin-like growth factor-II can substitute for insulin in the support of serum-free growth of EC cells (Nagarajan *et al*, 1982; Heath and Deller, 1983). Two forms, both derived from BRL culture medium, were used in this study; mature IGF-II (molecular weight 7,484), and a slightly larger precursor MSA-III-1 (CR-MSA) (molecular weight 8,700) (Moses *et al*, 1980b).

Platelet-derived growth factor (PDGF) is an acid-and heat-stable mitogen of molecular weight 30,000 (Antoniades *et al*, 1979; Heldin *et al*, 1979; Raines and Ross; 1982). It is not known whether this or a related factor is made by BRL cells, but if it were it would be expected to be present in the G-75 Fraction described in the previous Section. EC cells have been reported to produce PDGF-like growth factors (Gudas *et al*, 1983; Rizzino and Bowen-Pope, 1985).

Transforming growth factors are acid-and heat-stable polypeptides that reversibly induce non-neoplastic cells to express a transformed phenotype in

culture (DeLarco and Todaro, 1978b). They are of at least two types. The first, type α, are structurally related to epidermal growth factor (EGF) (Marquardt *et al*, 1983,1984). Their actions are believed to be mediated via the EGF receptor and they have biological activity indistinguishable from EGF *in vitro* (Massague, 1983). Murine epidermal growth factor was employed as a representative of the TGF-α family in this study. Type β transforming growth factor (TGF-β) is a homodimer of disulphide-linked polypeptide chains, each of molecular weight 12,500 (Frolik *et al*, 1983; Assoian *et al*, 1983; Roberts *et al*, 1983; Derynck *et al*, 1985). It binds to a unique cell surface receptor (Tucker *et al*, 1984b; Massague and Like, 1985). TGF-β can either promote or inhibit cellular growth (Roberts *et al*, 1985a; Sporn and Roberts, 1985; Hill *et al*, in press) depending on cell type and age, and also on the overall growth conditions. TGF-β may interact synergistically or antagonistically with other growth factors (Roberts *et al*, 1985a). In addition some of the actions of TGF-β are antagonised by retinoic acid (Brinckerhoff, 1983; Roberts *et al*, 1985b). Moreover, in contrast to IGF-II and PDGF, detectable levels of TGF-β are not present in calf serum (Massague *et al*, 1985), which is of note given that differentiation of EC cells occurs in the presence of 10% calf serum.

Recently it has been shown that BRL cells produce TGF-β (Massague *et al*, 1985), though not TGF-α. TGF-β is anomalously retarded by the Bio-Gel chromatography matrix (Massague *et al*, 1985), however, so that even though it has a molecular weight of 24-25,000 it is not necessarily present in the G-75 Fraction purified in Section 6.2. Embryonal carcinoma cells also produce a transforming growth factor activity (Rizzino *et al*, 1983). This appears to be distinct from both type α and type β TGFs in terms of its bioactivity (Rizzino, 1983c). This factor has not been purified, so its relationship to TGF-α and TGF-β and to the PDGF-like activity (Rizzino and Bowen-Pope, 1985) and embryonal carcinoma-derived growth factor (ECDGF) (Heath and Isacke, 1984) produced by EC cells remains unclear.

The above factors, all purified to homogeneity, were assayed over a range of concentrations for the capacity to inhibit differentiation of feeder-free monolayer cultures of PSA4 in CM containing 10% (v/v) foetal calf serum (Section 2.10.2.) and to stimulate DNA synthesis in quiescent NRK 49F rat fibroblasts (Section 2.17.). The results are summarized in Table 6.4.

Table 6.4
Effects of Purified Growth Factors on Differentiation of PSA4 EC Cells and DNA Synthesis in NRK 49F Fibroblasts

Growth Factor	Production by BRL Cells	Concentration Range (ng/ml)	Mitogen Activity	Inhibition of Differentiation
Insulin	-	100-20,000	-	-
IGF-II	+[a,b]	20-400	+	-
CR-MSA	+[a]	10-200	+	-
PDGF	Not known	2-60	+	-
EGF	-[c]	100	+	-
TGF-β	+[c]	0.1-0.5	+	-
"	"	1-10	-	-

[a]Dulak and Temin. 1973a
[b]Marquardt *et al*, 1981
[c]Massague *et al*, 1985
For details see text.

Table 6.5
Combinations of Growth Factors Found to be Ineffective in Inhibition of PSA4 Differentiation

TGF-β	PDGF^	EGF	"PC13.5"*
0.1ng	1U	-	-
0.5ng	-	100ng	-
0.5ng	10ng	-	-
0.5ng	20ng	-	-
0.5ng	-	-	60%
0.5ng	1U	-	60%
1.0ng	0.5U	-	-
5.0ng	10ng	-	-
5.0ng	-	-	60%
5.0ng	10ng	-	60%
-	10ng	-	60%

*Medium conditioned by PC13.5 EC cells by incubation (30ml/75cm^2 flask) for 4 days with exponentially growing cells.
^Two preparations of PDGF, one partially purified, the other purified to homogeneity, were used; 1 unit (U) of partially purified PDGF is equivalent to 28ng of pure PDGF (Bioprocessing Data Sheet)

Assays for differentiation inhibiting activity were performed as described in Section 2.10.2. in a final incubation volume of 1.0ml.

With the exception of insulin, all the growth factors stimulated [^{3}H]-thymidine incorporation by NRK 49F rat fibroblasts (though this response to TGF-β was small and only manifest at concentrations below 1ng/ml).

None of the factors inhibited the spontaneous differentiation of PSA4 on

gelatinized substrata, however. Cell viability was not unduly affected by any of the agents and in all cases the EC cells underwent complete morphological differentiation within 7 days (Plate 6.2A).

To the data in Table 6.4 can be added the observation that medium conditioned by PC13.5 EC cells, which should contain ECDGF (Heath and Isacke, 1984), an unidentified transforming growth factor activity (Rizzino *et al*, 1983; Heath, 1983) and the PDGF-like factor of Rizzino and Bowen-Pope (1985), has been shown in Section 5.2. not to inhibit spontaneous differentiation of PSA4.

In view of reports that responses to TGF-β are dependent on interactions with other growth factors (Roberts *et al*, 1985a; Sporn and Roberts, 1985) and the possibility presented by attempted purification of DIA that it might represent the concerted action of two mitogens (Section 6.2.5.), various combinations of factors were tested for their effect on PSA4 differentiation. None showed any indication of inhibitory action (Table 6.5).

6.3.2. Conditioned Media

Media conditoned by several cell lines were screened for the capacity to reproduce the effect of BRL-conditioned medium on monolayer cultures of PSA4. Four cell lines were chosen on the basis either of their production or lack of production of particular growth factors, or because they are thought to be equivalent to embryonic derivatives of the inner cell mass (ICM) and thus to be developmentally closely related to EC cells.

BRL-3A2 cells are a subclone of the original isolate of Buffalo rat liver (BRL) cells which do not produce detectable amounts of IGF-II (Nissley *et al*, 1977). BSC-1 is a continuous epithelial cell line derived from African green monkey kidney (Hopps *et al*, 1963). It produces a growth inhibiting factor which has recently been shown to be closely related to, if not identical with, TGF-β (Holley *et al*, 1978; Holley *et al*, 1980; Holley *et al*, 1983; Holley *et al*, 1984). PYS (Lehman *et al*, 1974) and PSA5E (Adamson *et al*, 1977), are two teratocarcinoma-derived cell lines which closely resemble extraembryonic endoderm. PYS is parietal endoderm-like (Hogan, 1980; Hogan *et al*, 1981) and PSA5E is visceral endoderm-like (Adamson *et al*, 1977; Hogan, 1980; Adamson, 1982).

Medium was conditioned following the standard protocol used for BRL cells (Section 2.7.), except that incubation was for only 48 hours in the case of PYS and PSA5E cells as these cultures rapidly acidified the medium. Even after 48 hours these media were quite acidic and were therefore neutralized with 1M NaOH prior to use. The media were assayed for the capacity to inhibit the spontaneous differentiation of PSA4 cultured in the absence of feeders in 25cm^2 flasks (Section 2.10.2.). The results are summarized in Table 6.6.

Table 6.6
The Effects of Medium Conditioned by Various Cell Lines on the Growth and Differentiation of PSA4 Monolayers

Cell Line	Final Concentration*	Growth Rate	Inhibition of Differentiation
BRL	100	Arrested	+++
"	60	Rapid	+++
BRL-3A2	100	Arrested	+++
"	60	Rapid	+++
BSC-1	75	Rapid	++
"	60	Rapid	+
PYS	70	Intermediate	+
"	60	Rapid	+
PSA5E	60	Poor^	-

*% (v/v) conditioned medium in final culture medium
^High level of cell death

Cultures,previously passaged at least once in the absence of feeders,were seeded at a density of $4x10^3/cm^2$ on gelatinized substrata in the medium under test (Day 1). They were refed with the same medium on Day 4. Differentiation was assessed by morphological criteria on Day 7:-

+++ less than 5% of cells differentiated
++ 5-10% of cells differentiated
+ 10-20% of cells differentiated

An estimate of growth was obtained from the surface area occupied by the cultures, with due allowance for the higher density of undifferentiated cells:-

Arrested - no increase in the size of individual colonies after Day 4
Poor - very sparse cultures but some progressive growth
Intermediate - cultures semi-confluent by Day 7
Rapid - cultures confluent (>$5x10^5$cells/cm^2) by Day 7

Medium conditioned by BRL-3A2 cells appeared indistinguishable from standard BRL-conditioned medium in terms of its effects on feeder-free monolayer cultures of PSA4. In 60:40 (v/v) BRL-3A2 conditioned medium:normal CM, PSA4 EC cells proliferated as a homogeneous undifferentiated population (Plate 6.2B).

Medium conditioned by BSC-1 cells also prevented differentiation, though not as effectively as BRL-or BRL-3A2 conditioned medium. Feeder-free cultures of PSA4 have been maintained for 4 serial passages in 75% BSC-1-conditioned medium during which time the proportion of morphologically differentiated cells never rose above 10%. Higher concentrations than 75% (v/v) were not investigated because after 3 days of conditioning the medium is fairly acid, indicative of a significant level of metabolic activity by the BSC-1 cells and a consequent depletion of nutrients and possible production of toxic substances.

The latter argument also applies to PYS- and PSA5E-conditioned media and probably accounts for the poorer growth observed. Medium conditioned by PYS cells did greatly reduce the level of spontaneous differentiation relative to control cultures in CM and this effect persisted after subculturing. Although over 80% of cells remained EC-like, there was a significant level of differentiation, comparable to that obtained in STO-feeder-conditioned medium (Smith and Hooper, 1983; see Section 5.2). Due to the high level of cell mortality in PSA5E-conditioned medium, its effects on differentiation are somewhat obscured. Most of the surviving cells did undergo morphological differentiation, however, suggesting that this medium contains little if any inhibitory activity.

Medium conditioned by BRL-3A2 cells was also shown to stimulate [^{3}H]-thymidine incorporation by NRK 49F cells after concentration and dialysis against 0.1M acetic acid (Table 6.7).

Table 6.7
Growth-Promoting Activity of Acid-Dialysed BRL-3A2-Conditioned Medium on NRK 49F Cells

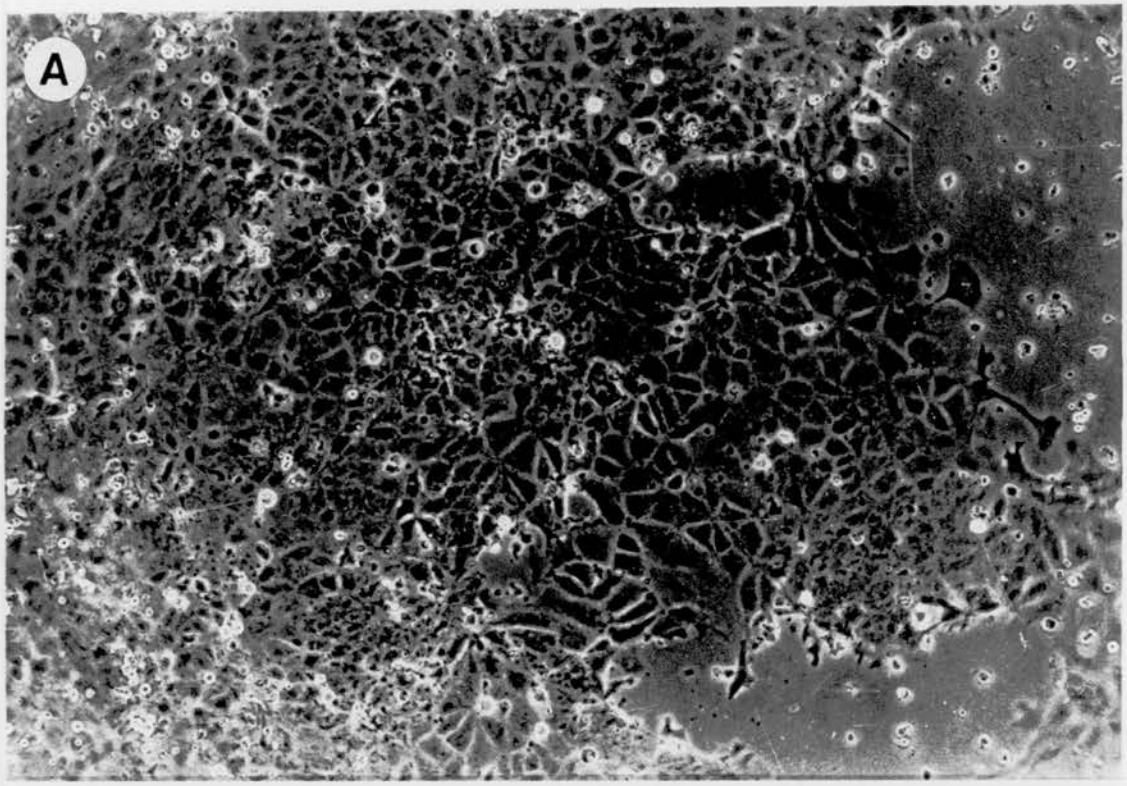
Sample	C.P.M.±s.e.m.
MSA buffer	36,709±6,348
Foetal calf serum	322,764±6,086
BRL-3A2 dialysate	124,806±17,838

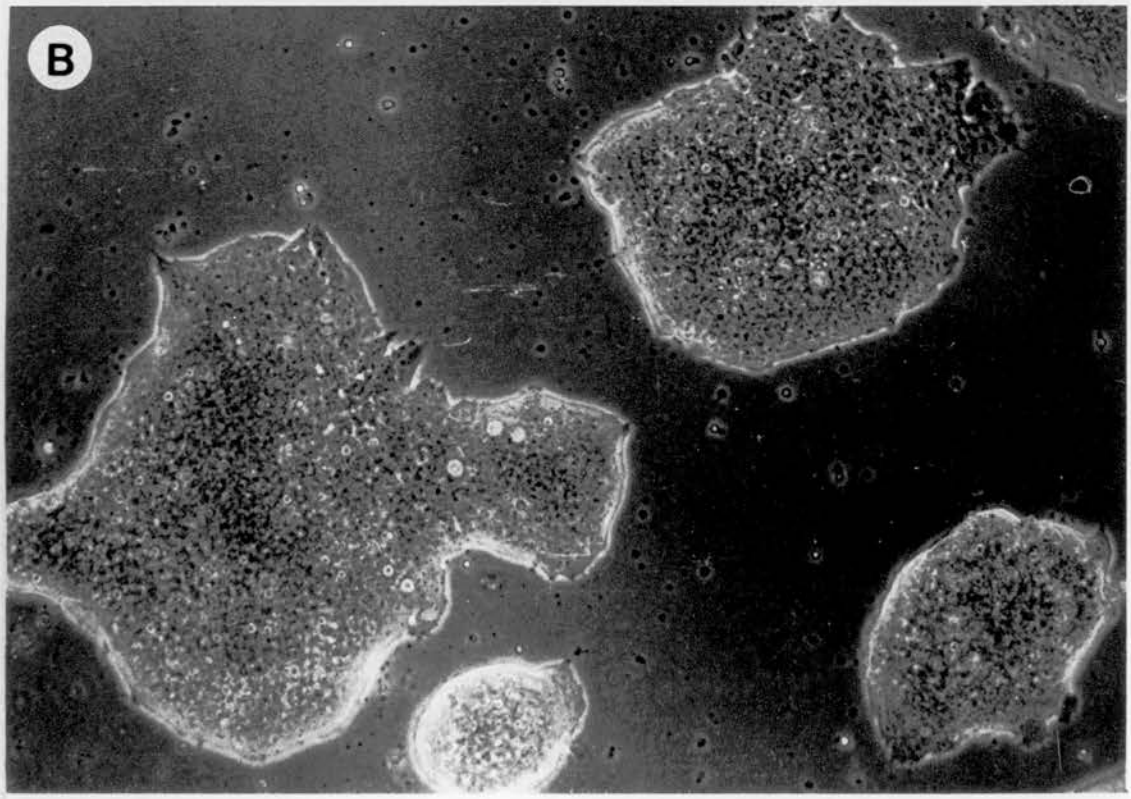
Serum-free conditioned medium (100ml) was lyophilized, resuspended in 2ml MSA buffer, dialysed against 0.1M acetic acid and clarified by high-speed centrifugation. After lyophilization, it was reconstituted in 0.05ml of 0.1M acetic acid and made up to 1ml with MSA buffer. Assays were performed in triplicate on 0.1ml samples as described in Section 2.17.

Plate 6.2 Feeder-Free Culture of PSA4 in CM Supplemented with IGF-II and in BRL-3A2-Conditioned Medium

Phase contrast

	Magnification
A. CM containing 50ng/ml IGF-II, 4 days	110
B. 60% (v/v) BRL-3A2-conditioned CM, second passage	121


A


B

6.3.3. Summary

The findings of Section 6.3.1. confirm the indications from partial purification of DIA that the activity does not reside in mature IGF-II (Plate 6.2A). Nor can it be reproduced by insulin. This conclusion can apparently be extended to preclude the involvement of any precursor forms of IGF-II, since medium conditioned by BRL-3A2 cells, which does not contain any biologically active IGF-II (Nissley *et al*, 1977), supports the feeder-free proliferation of PSA4 EC cells as efficiently as medium conditioned by IGF-II-producing BRL cells. It is noteworthy, however, that acid-dialysed extracts of BRL-3A2-conditioned medium do stimulate DNA synthesis by NRK 49F fibroblasts. This may be due to some other growth factor, possibly TGF-β, though it is also conceivable that this stock of BRL-3A2 cells has spontaneously resumed production of IGF-II. The latter possibility could be examined by fractionation of the conditioned medium followed by competitive binding assays with radio-iodinated IGF-II (Moses *et al*, 1980a).

Several other growth factors, all of which stimulated DNA synthesis by NRK 49F rat fibroblasts to varying degrees, have also been shown to have no effect on PSA4 differentiation. This is predictable for EGF which is known not to be produced by BRL cells and for which EC cells lack functional receptors (Rees *et al*, 1979). It is not known whether the line of BRL cells used in this study produce PDGF or some related mitogen. EC cells apparently do produce PDGF-like activity but they do not bind exogenously added PDGF (Gudas *et al*, 1983; Rizzino and Bowen-Pope, 1985), indicating either that they lack receptors or that their receptors are down-regulated. Consequently it is not surprising that PDGF fails to elicit any change in differentiation of PSA4.

TGF-β is present in medium conditioned by BRL cells (Massague *et al*, 1985). It is also produced by mouse embryos and mouse embryo cell lines (Proper *et al*, 1982; Tucker *et al*, 1984a,b) and there is evidence that it may play a major regulatory role in foetal development (Lawrence *et al*, 1984; Hill *et al*, in press). Medium conditioned by BSC-1 cells contains a TGF-β-like factor (Holley *et al*, 1984) and is shown in Section 6.3.2. to inhibit the spontaneous differentiation of PSA4. Embryonal carcinoma cells themselves release a transforming growth factor activity, though this appears to be biologically distinct from TGF-β (Rizzino *et al*, 1983; Rizzino, 1983c). It is not known whether EC cells express

cell surface receptors for TGF-β. Over a wide concentration range (0.1-10ng/ml), however, purified TGF-β has no discernible effects on the differentiation of PSA4.

Extraembryonic endoderm is the first differentiated derivative of the inner cell mass (ICM) formed in the mammalian embryo (Section 1.2.). It is therefore a potential source of DIA *in vivo*. The visceral endoderm-like PSA5E cell line does not appear to produce significant DIA activity. In contrast medium conditioned by the parietal endoderm cell line PYS does exhibit Differentiation Inhibiting Activity. PYS-conditioned medium is not as potent an inhibitor of PSA4 differentiation as medium conditioned by BRL cells, however. This could be because PYS cells produce less DIA than BRL cells, or because its effects are counteracted by deterioration of the medium due to the active metabolism of these cells. Another possibility is that the effects of DIA might be antagonised by other factors produced by these cells. PYS cells produce laminin (Hogan et al, 1980; Dziadek and Timpl, 1985), for example, which has been shown to induce EC cell differentiation into endoderm (Rizzino, 1983b; Adamson and Grover, 1983). Koopman and Cotton (1984) have also reported that medium conditioned by PYS cells partially inhibits differentiation of EC cells. This observation suggests that DIA may be produced by the parietal endoderm *in vivo*.

6.4. DISCUSSION

The non-dialysable activity produced by BRL cells which inhibits differentiation of feeder-dependent EC cells has been named Differentiation Inhibiting Activity (DIA). This activity is not significantly affected by frozen storage, lyophilization or acidification, thus facilitating routine fractionation procedures. The attempt to purify and identify the factor or factors responsible has proved only partly successful, however.

DIA can be resolved from both IGF-II and the bulk protein present in BRL-conditioned medium by gel filtration at acid pH. It is eluted from a Sephadex G-75 column as a single region of activity with an apparent molecular weight of 20-35,000. This region is mitogenically active. It contains several proteins (Plate 6.1), however, and both the nature of the mitogen or mitogens and their relationship to DIA remains unclear. Clarification of this

issue and purification of DIA to homogeneity requires the application of a high resolution technique such as high performance liquid chromatography. Given the stability of DIA and the greater than 6-fold purification achieved by gel filtration, it may prove relatively straightforward to obtain it in pure form using such advanced methods.

DIA is not mature IGF-II, nor apparently any precursor thereof. In retrospect this is not surprising because serum contains high endogenous levels of IGFs (Moses *et al*, 1980a; Stiles *et al*, 1980; Massague *et al*, 1985). Thus most physiological responses are manifest in medium containing 10% (v/v) calf serum as used here.

However, the elution of DIA from a G-75 column within a broad region of mitogenic activity (Fig.6.2A), which displays similar thermal stability (Table 6.3), suggests that it could be some other growth factor. This is not refuted by the presence of DIA in BRL-3A2-conditioned medium as this also contains a mitogen which stimulates DNA synthesis by quiescent NRK 49F fibroblasts (Table 6.5). Not unexpectedly (see Section 6.3.3.), neither EGF nor PDGF exhibit Differentiation Inhibiting Activity. A stronger candidate appeared to be TGF-β which is not present in calf serum and is known to be synthesized by BRL cells. This factor, in conjunction with other activities present in serum, reversibly induces in non-transformed cells the capacity for anchorage-independent growth (Roberts *et al*, 1983). Under appropriate conditions it also promotes DNA synthesis by NRK 49F fibroblasts (Massague and Like, 1985). Undifferentiated EC cells are capable of anchorage-independent growth in semi-solid media (Heath *et al*, 1981; Heath, 1983) and feeder-independent EC cells produce a transforming growth factor which might function in an autocrine manner (Rizzino *et al*, 1983; Heath, 1983; see Section 1.7.). However, purified human TGF-β, which is biologically active on mouse cells and binds with high affinity to specific cell surface receptors on mouse embryo-derived cells (Tucker *et al*, 1984b; Massague and Like, 1985), has no effect on the spontaneous differentiation of PSA4. Nor, as was noted in the previous Chapter (Section 5.2.), does the feeder-independent PC13.5 EC cell line release DIA into the culture medium, arguing against the autocrine hypothesis.

It is possible therefore that DIA is a hitherto unidentified growth factor. On the other hand, there is no *a priori* reason for DIA to be mitogenic. In fact the observation of arrested growth of PSA4 at high concentrations of

BRL-conditioned medium (Section 5.2.) suggests that it may even possess growth inhibitory properties.

Of the proteins present in the G-75 Fraction, which represents almost a 7-fold purification of DIA, only one can be identified with any degree of certainty (Section 6.2.6.). This is the 30,000 molecular weight IGF-II carrier protein which is also produced by BRL-3A2 cells. It is difficult to envisage how this could function as DIA as it is present in excess in serum and its only known function is to increase the half-life of IGF-II in the circulation (Knauer and Smith, 1980; Herrington *et al*, 1983). Moreover, it inhibits the biological activity of IGF-II (Knauer and Smith, 1980).

An entity produced by BRL cells which inhibits the differentiation of myoblasts has been described by Florini and co-workers (Evinger-Hodges *et al*, 1982; Florini, 1984; Florini *et al*, 1984). This so-called Differentiation Inhibitor (DI) reversibly inhibits all aspects of myogenesis in myoblasts from a variety of sources. It has not been purified to homogeneity but partial purification indicates that it is a relatively heat-stable protein of apparent molecular weight 30-36,000 which is resolved from IGF-II by Sephadex G-75 gel filtration in acetic acid. It appears to be biologically distinct from the IGF-II carrier protein. DI is not mitogenic for myoblasts and its growth-promoting capacity on other cell types has not been investigated. This factor could be present in the G-75 Fraction purified in Section 6.2. Consequently it would be of interest to examine the effects of this Fraction on myogenesis and to investigate the relationship between DI and DIA.

The finding that inhibition of PSA4 differentiation by BRL-conditioned medium is not due to IGF-II does not preclude an involvement of this factor in some of the phenomena reported in the previous Chapter. It is quite possible, for example, that the apparent enhancement of endodermal proliferation described in Section 5.3.4. is due to the elevated level of IGF-II rather than to a direct effect of DIA. The parietal endoderm-like cell line PYS expresses cell surface receptors for IGF-II (Anderson et al, 1983a) suggesting that parietal endoderm might be responsive to this growth factor. Extraembryonic endoderm may also produce IGF-II (Anderson et al, 1983b). The alternative type of differentiation adopted by PSA4 and B2B2 in BRL-medium when exposed to retinoic acid (Section 5.2.6.) is unlikely to arise from an interaction of the EC cells with IGF-II during the process of commitment, however, since the purified factor has no

obvious effect on the form of spontaneous differentiation in CM (Plate 6.2a). Possible interactions between DIA and IGF-II can only be fully investigated following further purification of the former. In the meantime, however, it would be of considerable interest to compare embryoid body development in medium conditioned by BRL and BRL-3A2 cells. Any effect due to IGF-II should not be manifest in the latter unless the growth factor is exogenously provided.

It was proposed in Chapter 5 that the Differentiation Inhibiting Activity present in BRL-conditioned medium may play an important role in early embryonic development (Section 5.4.). The spontaneous differentiation of PSA4 and B2B2 in the absence of feeders, and the failure of medium conditioned by PC13.5 EC cells to inhibit this process (Section 5.2.), indicates that DIA is not produced by EC cells themselves or their embryonic counterparts. Consequently if DIA is present in the implantation stage embryo, it must be synthesized by either the trophectoderm or the extraembryonic endoderm. Therefore the finding that parietal endoderm-like PYS cells produce DIA-like activity is of great significance. It is important to establish whether the observations on medium conditioned by PYS and PSA5E cell lines can be repeated using parietal and visceral endoderm isolated directly from embryos. The possible synthesis of DIA by trophectoderm should likewise be examined using authentic embryo-derived tissue. Whether DIA is produced by primitive endoderm might be investigated using the primitive endoderm-like cell line 1H5 isolated by Kahan and Adamson (1983) or possibly the Dif 5 endodermal cell line derived by Nagarajan *et al*, (1983a,b).

CHAPTER 7

THE REQUIREMENT OF 2-MERCAPTOETHANOL FOR FEEDER-FREE GROWTH OF PSA4-DERIVED EMBRYONAL CARCINOMA CELLS

7.1. INTRODUCTION

Initial reports (Martin and Evans, 1975a,b) on the growth of feeder-dependent EC lines in the absence of feeders indicated that it was not possible to maintain monolayer cultures, even at high density, as the cells died after one or two serial passages. Only aggregates of EC cells survived under these conditions. These observations were interpreted as implying that feeder cells, in addition to providing a suitable growth surface and preventing differentiation, were also essential for viability of feeder-dependent EC cells.

Subsequently, Oshima (1978) reported that the viability of these cell lines could be sustained in the absence of feeders if 2-mercaptoethanol was present in the medium. Under these conditions extensive differentiation occurs. Smith and Hooper (1983) showed that differentiation was reduced in medium conditioned by incubation with STO feeders, enabling the maintenance of largely undifferentiated EC cell populations in the absence of feeders. These experiments were performed in medium supplemented with 10^{-4}M 2-mercaptoethanol. The presence of this thiol compound was proposed to account for the apparent contradiction with earlier observations (Martin and Evans, 1975a) that conditioned medium could not substitute for feeder layers in the culture of feeder-dependent EC lines. This proposition was not directly investigated, however.

The experiments reported in Section 7.2 represent such an investigation into the role of 2-mercaptoethanol in the growth and differentiation of feeder-dependent EC cells plated on gelatinised substrata. In Section 7.3. the possible function of junctional communication between EC cells and feeders in sustaining viability of the former in the absence of 2-mercaptoethanol is examined.

7.2. THE ROLE OF 2-MERCAPTOETHANOL IN CONDITIONED MEDIUM

Previous work on STO feeder-conditioned medium in this laboratory (Smith and Hooper, 1983; Smith, 1984; Chapters 3, 4 and 5 above) has employed CM containing 10^{-4}M 2-mercaptoethanol. Likewise the experiments reported in Chapter 5 on medium conditioned by BRL cells were performed in the presence of 2-mercaptoethanol (apart from the induction of embryoid bodies). To examine whether the thiol plays any essential role in the maintenance of cell viability and/or inhibition of differentiation, plating tests were performed on PSA4TG12 (Slack *et al*, 1977) in the absence of feeders in various media with and without 2-mercaptoethanol. The results are presented in Table 7.1.

Table 7.1
Colony Formation by PSA4TG12 on Gelatinised Substrata in the Presence or Absence of 2-Mercaptoethanol

Conditioning	Addition	Number of Colonies Expt.I	Expt.II	Differentiation
None	-	0	0	
"	2-me[a]	114.0	178.0	+
"	Catalase	0	0	
Feeders	-	0	ND[b]	
"	2-me	138.5	ND	±
"	Catalase	0	ND	
BRL Cells	-	0	0	
"	2-me	139.5	258.5	-
"	P[c], 2-me	ND	183.5	-
"	Catalase	ND	0	

[a]2-mercaptoethanol
[b]Not determined
[c]2-mercaptoethanol added after conditioning

Data are the means of plating tests performed in duplicate as described in Section 2.9. on PSA4TG12 cultures previously passaged in BRL-medium in the absence of feeders. Cells were plated in BRL-medium containing 10^{-4}M 2-mercaptoethanol. After 24 hours this was changed to normal medium containing 10% (v/v) foetal calf serum but lacking 2-mercaptoethanol, except where specified. 2-mercaptoethanol was present at a concentration of 10^{-4}M and catalase at 5µg/ml where indicated. Feeder-conditioned medium was used undiluted; BRL-conditioned medium was diluted 60:40 with CM. Cultures were fixed and stained with Leishmans' after 6 days. Differentiation was assessed morphologically:-

+ over 90% of colonies wholly differentiated
± over 75% of colonies contain a mixture of EC and differentiated cells
- over 90% of colonies wholly EC

It is apparent that colony-formation in the absence of feeders is absolutely dependent on the presence of 2-mercaptoethanol, regardless of any effect of conditioned medium. The methodology precludes the involvement of 2-mercaptoethanol solely in the initial attachment of EC cells to the substratum and demonstrates that it is required for survival of attached cells.

Oshima (1978) proposes that the function of 2-mercaptoethanol is the reductive modification of the sulphydryl content of normal medium. This could be necessary to prevent the formation of toxic free radicals generated by autoxidation of cysteine (Saez *et al*, 1982). The latter process is also inhibited by catalase. The addition of catalase (5µg/ml) to culture medium has no effect on cloning efficiency of PSA4TG12, however. This indicates that the principal role of 2-mercaptoethanol is unlikely to be one of detoxification via inhibition of the production of free radicals from sulphydryl groups.

2-mercaptoethanol is required by both differentiating and non-differentiating cultures indicating that its primary effect is not on the differentiation process (see also following Section). It remains possible, nonetheless, that it may have a secondary effect in the inhibition of differentiation by conditioned medium. This possibility cannot be directly examined using monolayer cultures of feeder-dependent EC cells. Feeder-independent EC cell lines, such as PC13.5 (Hooper and Slack, 1977) do not require 2-mercaptoethanol for proliferation, however. Consequently its role in the inhibition of retinoic acid-induced differentiation of PC13.5 by BRL-conditioned medium (Section 5.3.5.) can be investigated. This was carried out employing the fibrin overlay assay for plasminogen activator production (Section 2.10.5.) as a measure of differentiation. The results are presented in Table 7.2.

The proportion of colonies producing plasminogen activator is slightly decreased in the absence of 2-mercaptoethanol in both normal and conditioned medium. This probably arises because of the smaller size of the colonies found in all media in the absence of the thiol, which reduces the detectability of the assay. An alternative explanation may be that 2-mercaptoethanol slightly enhances the induction of differentiation by retinoic acid, though there is no direct evidenc for this. It is clear, however, that 2-mercaptoethanol is not required for the inhibitory action exerted by BRL-conditioned medium.

Table 7.2
Inhibition of Retinoic Acid-Induced Differentiation of PC13.5 by BRL-Conditioned Medium in the Presence or Absence of 10^{-4}M 2-Mercaptoethanol

Medium	2-me[b]	RA[c]	% Colonies Producing PA[a] Expt.I	Expt.II
CM	+	-	0.8	0.5
"	+	+	85.4	77.5
"	-	+	80.6	70.5
100% BRL[d]	+	-	0.4	ND[e]
"	+	+	7.7	ND
"	-	+	2.6	ND
60% BRL	+	-	ND	0.2
"	+	+	42.5	46.7
"	-	+	ND	37.2

[a]Plasminogen activator
[b]10^{-4}M 2-mercaptoethanol
[c]10^{-7}M retinoic acid
[d]Undiluted BRL-conditioned medium; colonies very small, even in the presence of 2-mercaptoethanol
[e]Not determined
Fibrin overlay assays were performed as described in Section 2.10.5. six days after plating.

7.3. THE ROLE OF JUNCTIONAL COMMUNICATION BETWEEN FEEDERS AND EC CELLS IN THE ABSENCE OF 2-MERCAPTOETHANOL

The growth of EC cells on feeder layers is not dependent on the presence of 2-mercaptoethanol (Martin and Evans, 1975a,b; Oshima, 1978). The finding that feeder-conditioned medium must be supplemented with 2-mercaptoethanol in order to sustain the viability of feeder-dependent EC cells implies that at least one effect of the feeder layers is not mediated via production of stable diffusible factors. This effect could be dependent on some unstable entity produced by the feeder cells, or might require intercellular communication between feeders and EC cells. The latter explanation raises the possibility of the involvement of metabolic cooperation in the maintenance of EC cell survival by feeders.

It has been proposed that the intercellular transfer of small molecules via gap junctions may be of considerable metabolic and possibly also developmental importance (reviewed by Hooper and Subak-Sharpe, 1981; see Section 1.8.). EC cells form functional gap junctions with feeder cells (Hooper and Morgan, 1979) and these may permit the transfer of some essential metabolite(s). Such

metabolites could be produced exclusively by the feeders or might be obtained from serum by some uptake mechanism lacking in feeder-dependent EC cells. A possible function of 2-mercaptoethanol is to modify some serum component(s) in such a way as to render it available to EC cells (see following Section for further discussion).

The role of junctional communication between EC cells and feeders in maintaining viability of the former was investigated by comparing colony formation by PSA4TG12 EC cells on feeder layers formed from metabolic cooperation-competent (mec^+) and metabolic cooperation-deficient (mec^-) fibroblasts in the presence and absence of 2-mercaptoethanol (Table 7.3). STO mouse embryo fibroblasts (Martin and Evans, 1975b) and Don Chinese hamster fibroblasts (Hsu and Zenzes, 1964) were used to prepare mec^+ feeder layers. Slack *et al* (1978) described a clone of Don 6 cells which showed a reduced grain count index when tested as a recipient of [^{3}H]-hypoxanthine-derived nucleotides from the EC cell lines PC13TG8 and R5/3. This clone, designated Don 6, also shows a reduced grain count index in homotypic uridine nucleotide transfer assays compared with Don cells, indicating a deficiency in the capacity to form gap junctions (M.L.Hooper, personal communication). Mec^- feeders were prepared from this variant. All three feeder layers inhibited differentiation such that over 90% of colonies consisted wholly of morphologically undifferentiated EC cells.

Table 7.3
Colony Formation by PSA4TG12 on Mec^+ and Mec^- Feeder Layers in the Presence and Absence of 2-Mercaptoethanol

Feeders	2-Me*	No. of Colonies Expt.I	Expt.II	% Mean Relative Cloning Efficiency^
STO	+	632.0	270.7	-
"	-	628.5	270.0	99.55
Don	+	651.5	318.3	-
"	-	656.0	237.0	87.60
Don 6	+	720.5	311.3	-
"	-	635.0	256.3	85.23

*10^{-4}M 2-mercaptoethanol
^Colonies formed in the absence of 2-mercaptoethanol as a percentage of colonies formed in its presence

Plating tests were performed in duplicate (Expt.I) or triplicate (Expt.II) as described in Section 2.9.

The cloning efficiency of PSA4TG12 cells on feeder layers is only slightly decreased in the absence of 2-mercaptoethanol. Nor is there any effect on differentiation. There is no significant difference in either respect between Don and Don 6 feeder layers. Although this suggests that junctional communication may not be a necessary component of the feeder effect on EC cell viability, the size of the colonies provides an indication to the contrary. After 6 days in the medium under test, colonies formed on STO or Don feeders in the absence of 2-mercaptoethanol appear to be on average about half the size of colonies formed in the presence of the thiol. Similar observations were reported by Oshima (1978). Colonies grown on Don 6 feeders in medium containing 2-mercaptoethanol are comparable in size to those grown on Don feeder layers. In the absence of 2-mercaptoethanol, however, colonies on Don 6 feeders are only 5% to 20% of this size, i.e less than half the size of colonies formed on Don feeders under the same conditions.

In the absence of differentiation, EC colony volume is determined by the number of individual EC cells present (assuming a relatively constant cell volume) which is dependent on the growth rate. These observations therefore suggest that metabolic cooperation with feeders could be limiting for EC cell growth in medium lacking 2-mercaptoethanol. This possibility was examined further using the mec^- derivative of PSA4TG12, PT2md1 (Smith, 1984). Like other PSA4 derivatives this cell line has a cloning efficiency of zero in the absence of 2-mercapotoethanol. As this is a non-differentiating clone (see Chapter 4) this provides further evidence that the requirement for 2-mercaptoethanol is unrelated to the differentiation process. The results of a comparison of colony formation by PT2 and PT2md1 on either mec^+ Don or mec^- Don 6 feeders in the presence and absence of 2-mercaptoethanol are presented in Table 7.4. In this experiment there is a marked decrease in cloning efficiency of PT2md1 on Don 6 feeders in the absence of 2-mercaptoethanol. This is the combination expected to cooperate least well, so the result is consistent with an involvement of junctional communication.

This approach was extended to take into account the differences in colony size. The areas occupied by 20 randomly selected colonies from each dish were measured using an IBAS-2 image analyser and a mean colony area obtained. This operation was performed on the dishes from the experiment reported in Table 7.4, which were incubated in test medium for 6 days, and on dishes from a separate experiment incubated in test medium for 4 days.

Table 7.4
Colony Formation by PT2 and PT2mdl on Mec^+ and Mec^- Feeder Layers in the Presence and Absence of 10^{-4}M 2-Mercaptoethanol

	PT2		PT2mdl	
2-mercaptoethanol	Don	Don 6	Don	Don 6
+	830	724	402	361
-	809	544	349	171
-/+ x100%*	97.5	75.1	86.8	47.4

*Equivalent to relative cloning efficiency in Table 7.3
Plating tests were performed as detailed in Section 2.9. Data are the mean numbers of colonies formed per dish (1 to 3 dishes scored at each point).

Table 7.5
Values of α_i for PT2 and PT2mdl on Mec^+ and Mec^- Feeder Layers in the Presence and Absence of 10^{-4}M 2-Mercaptoethanol

	PT2		PT2mdl	
2-mercaptoethanol	Don	Don 6	Don	Don 6
+	.6717	.5643	.5770	.5325
-	.4838	.2421	.3468	.1794
-/+ x100%	72.0	42.9	60.1	33.7

For details including definition of α_i see text.

Assuming that surface area occupied is proportional to the number of EC cells present in non-differentiating cultures*, the change in colony area with time is determined by cell growth rate. Therefore, assuming a constant growth rate for each combination of cells and medium, the culture surface area occupied by a colony should fit an exponential of the form:-

$$A=A_o e^{\alpha_i t}$$

where A=area

A_o=constant (area occupied at time 0)

α_i=growth constant for the ith combination of cells and medium

t=time (days in test medium)

*Note that the validity of this model does not depend upon the assumption of proportionality between surface area and cell numbers. It applies equally to any situation where the area is proportional to some power n of the cell number. This would be the case, for instance, if the mean depth of a colony were proportional to its radius, n being 2/3 under such conditions.

The combined data from the two experiments (1 to 3 dishes scored for each point in both experiments) were fitted to this model by regression analysis using the statistical software package GLIM (Dobson, 1983). The values for α_i are tabulated in Table 7.5.

The value of α_i is significantly greater in the presence of 2-mercaptoethanol than in its absence (F test, $P<0.05$) for all four cell combinations. These data thus confirm that 2-mercaptoethanol enhances EC cell growth on feeders.

The ratio (α_i in the absence of 2-mercaptoethanol/α_i in the presence of 2-mercaptoethanol) is much lower for both PT2 and PT2md1 on mec^- Don 6 feeders compared with mec^+ Don feeders. This finding supports the indication from reduced cloning efficiency of PT2md1 (Table 7.4) that junctional communication with feeders facilitates the survival and proliferation EC cells in the absence of 2-mercaptoethanol. The significance of differences in this ratio between PT2 and PT2md1 are unclear, however, as PT2 has a significantly higher value of α_i than PT2md1 when cultured in the absence of feeders in BRL-medium containing 2-mercaptoethanol (F test, $P<0.05$). Nonetheless it is noteworthy that the trend is the same as that found for cloning efficiency (Table 7.4) and that the lowest α_i ratio is produced by the combination of mec^- PT2md1 on mec^- Don 6 feeders.

7.4. DISCUSSION

The results presented in Section 7.2 establish that the presence of 2-mercaptoethanol is essential for survival of PSA4-derived EC cells at low density in the absence of feeders, even in medium conditioned by feeders or BRL cells. Koopman and Cotton (1984) have also reported a requirement for 2-mercaptoethanol in feeder-conditioned medium to support feeder-free growth of the NG2 EC cell line. The maintenance of both differentiating and non-differentiating feeder-dependent EC monolayers on gelatinised substrata thus depends on the presence of 2-mercaptoethanol. Differentiated derivatives of EC cells apparently do not require this thiol compound, however (Oshima, 1978).

Evidence is also presented in Section 7.2 that 2-mercaptoethanol does not have a secondary role in the differentiation inhibiting activity of conditioned medium. To the findings on retinoic acid-induced differentiation of PC13.5

reported in Table 7.2 can be added observations on embryoid body formation in BRL-conditioned EfC10 (Section 5.3.4.). EfC10 does not contain 2-mercaptoethanol, yet BRL-conditioned EfC10 inhibits cavity formation by embryoid bodies. Thus two of the three differentiation-inhibiting actions of BRL-conditioned medium reported in Chapter 5 do not require 2-mercaptoethanol. If, as is probable, all three actions arise from the same activity (DIA), it is unlikely that 2-mercaptoethanol is involved in the third, namely inhibition of spontaneous differentiation in the absence of feeders. Unfortunately this cannot be tested directly as 2-mercaptoethanol is necessary to sustain cell viability under such conditions. Only when the mechanism of the latter effect has been resolved and alternatives developed, might it prove possible to formally preclude an involvement of 2-mercaptoethanol in this form of inhibition of differentiation.

Catalase cannot replace 2-mercaptoethanol (Table 7.1), indicating that its role is probably not merely one of detoxification. This could be investigated further, however, by attempting substitution with mannitol, a molecule which prevents free radical formation and enhances the survival of PC13.5 EC cells in serum-free medium (J. Heath, personal communication).

Feeder cells can replace the requirement for 2-mercaptoethanol (although even in feeder cultures the thiol enhances EC cell growth, Table 7.5). The possibility that this effect is mediated via metabolic cooperation between feeders and EC cells is the subject of an investigation reported in Section 7.3. Preliminary indications from efficiency of colony formation by the mec^- EC line PT2md1 on mec^+ and mec^- feeders in the presence and absence of 2-mercaptoethanol and from growth of both mec^+ PT2 and mec^- PT2md1 under such conditions, are that junctional communication may be involved in this phenomenon. More data is needed to substantiate this conclusion, however. In particular the degree of metabolic cooperation between the various EC-feeder pairs should be quantified and the values correlated with those on cloning efficiency and growth.

An alternative approach would be to determine whether cell contact is necessary for the feeder effect. This could be examined by coculture experiments in which EC cells and feeders are cultured in close proximity in the same culture medium but are prevented from making physical contact (Isacke and Deller, 1983).

The mechanism of 2-mercaptoethanol action on EC cells remains uncertain. However, the sulphydryl dependence of certain haematopoietic cells *in vitro* (Toohey, 1975) provides a model which could account for the observations reported in this Chapter. Mercaptoethanol is necessary for the growth of primary explants of haematopoietic cells *in vitro* (Toohey, 1975). This requirement can be substituted by various thiol compounds at higher concentrations. Established cell lines, however, do not in general show such sulphydryl-dependence. It is notable in this regard that feeder-dependent EC cells require 2-mercaptoethanol for growth in the absence of feeders, whereas EC lines such as F9 and PC13, which are established to grow independently, do not exhibit this requirement.

The biochemical basis of the sulphydryl-dependence of primary haematopoietic cells has been elucidated by Toohey (1977,1978,1982; reviewed by Hooper, 1985). These cells lack the enzyme methylthioadenosine nucleoside phosphorylase (MTANP) which produces methylthioribose-1-phosphate from methyladenosine. The former is degraded to methyl mercaptan. This appears to be essential for cell division (Toohey and Cline, 1976). Consequently cells lacking the enzyme MTANP require an exogenous source of methyl mercaptan. This can be provided by serum, which contains disulphide-linked methyl mercaptan. In this case, however, a thiol carrier is required which can form a mixed disulphide with the methyl mercaptan, thus liberating it from serum, and transfer it across cell membranes (Eldjarn *et al*, 1962). Mercaptoethanol apparently functions as such a carrier in haematopoietic cell cultures.

The requirement of feeder-dependent EC cells for 2-mercaptoethanol in the absence of feeders could be accounted for if they lack MTANP and therefore need an exogenous supply of methyl mercaptan. This could be provided by serum in the presence of a thiol, or via metabolic cooperation with the feeders, which presumably possess MTANP as they grow in medium lacking 2-mercaptoethanol. The entity transferred via the gap junctions could be methyl mercaptan or some metabolite thereof, or the precursor methylthioribose-1-phosphate. This hypothesis could explain the enhanced growth of EC cells on feeders induced by 2-mercaptoethanol (Oshima, 1978; Section 7.3), as provision of an essential metabolite via junctional transfer alone would eventually become rate-limiting for EC growth.

If this hypothesis is correct, it should be possible to replace 2-mercaptoethanol

with various other sulphydryl compounds (Toohey, 1975) or mixed disulphides (Toohey and Cline, 1976) in the feeder-free culture of PSA4 and its derivatives. The definitive test, however, would be to demonstrate that feeder-dependent EC cells lack MTANP whilst feeder-independent EC cells and feeder cells possess active enzyme.

Finally it should be noted that even if the primary role of 2-mercaptoethanol is as a methylthio carrier, it may still have an important secondary function as a detoxificant and/or in providing a reducing environment. Thus, although 2-mercaptoethanol is not necessary for growth of PC13.5 cells in serum-containing medium, it does facilitate proliferation of these cells in serum-free medium supplemented with transferrin, insulin and partially purified fetuin (Rizzino and Sato, 1978). In completely defined medium, however, in which fetuin is replaced by fibronectin, 2-mercaptoethanol is no longer required (Rizzino and Crowley, 1980).

CHAPTER 8

DISCUSSION

Chapters 3 and 4 of this thesis are concerned with the effects of retinoic acid on wild-type and variant EC cells and attempt to clarify whether there is a single biological mode of action of this agent. The induction of EC cell differentiation by retinoic acid is well-established, though the mechanism by which this is effected remains unknown and its physiological significance is uncertain (Section 1.5.). Recent work in this laboratory (Smith, 1984) has shown that retinoic acid also inhibits metabolic cooperation between EC cells. The mechanism and biological significance of this effect are likewise obscure. However, junctional communication may be important in developmental signalling (Section 1.8.) and it has been postulated that the inhibition of metabolic cooperation could provide a unifying mechanism for the pleiotropic effects of retinoic acid (Maden, 1982; Pitts *et al*, in press).

Circumstantial support for this proposition comes from the finding that the structural requirements for inhibition of metabolic cooperation by analogues of retinoic acid (Pitts *et al*, in press) appear similar to those reported for other biological actions of retinoids, including the induction of differentiation of EC cells (Jetten and Jetten, 1979; Sherman *et al*, 1983b; Strickland *et al*, 1983). Thus all-*trans*-retinoic acid is slightly more potent than the 13-*cis* isomer and the terminal carboxyl group is essential, though not sufficient, for biological activity (see Fig.1.3). This is also the structural specificity exhibited by cellular retinoic acid binding protein (CRABP), the proposed intracellular mediator of retinoic acid effects in the steroid hormone model of retinoid action (Chytil and Ong, 1978; see Section 1.5.2.). Furthermore, inhibition of metabolic cooperation is rapid, occurring within minutes (Pitts *et al*, in press) and preceding other responses which often take place over days (Section 1.5.2.). Higher concentrations of retinoic acid appear to be necessary for inhibition of junctional communication than are required for many of its other biological effects, but Pitts *et al* (in press) have shown that cooperation between some cell types is sensitive to lower concentrations and argue that these may be the natural target cells. Furthermore, it has been shown that lower concentrations of retinoic acid can effectively inhibit junctional communication over a prolonged incubation period (Walder and Lutzelschwab, 1984). Therefore its

apparent concentration-dependence may not preclude the involvement of inhibited metabolic cooperation in the mediation of retinoic acid activity.

EC cells provide an appropriate means of investigating the possible relationship between inhibition of metabolic cooperation and induction of differentiation. Previous work in this laboratory (Smith, 1984) has shown that junctional transfer of nucleotides by a variety of EC cell lines is inhibited by retinoic acid at concentrations exceeding 10^{-5}M. The cell lines examined include the feeder-independent PC13TG8 (Hooper and Slack, 1977), a subclone of PC13. Differentiation of PC13TG8 is induced by concentrations of retinoic acid as low as 10^{-8}M, though higher concentrations (10^{-6}-10^{-5}M) are required for differentiation of the entire population. This difference in concentration-dependence suggests that the two phenomena may be independent but, as pointed out above, is not conclusive, particularly as the inhibition of metabolic cooperation is assayed over only 4 hours whereas maximal induction of differentiation requires at least 24 hour exposure to the retinoid (Rayner and Graham, 1982).

The experimental data presented in Sections 3.2.1. and 4.2. of this thesis provide more compelling evidence that retinoic acid-induced differentiation of EC cells is not mediated via inhibition of metabolic cooperation. In the former it is shown that the variant PTmr0, which is resistant to the effect on junctional communication (Smith *et al*, in press), is induced to differentiate by incubation with retinoic acid. A differentiation response is detectable in 10^{-8}M retinoic acid whereas metabolic cooperation appears completely unresponsive at the highest concentration tested (10^{-4}M) by the uridine nucleotide transfer assay (Smith *et al*, submitted). The ouabain rescue assays on PC13.5 reported in Section 4.2. demonstrate that prolonged incubation does not enhance the inhibitory effects of retinoic acid on junctional communication between EC cells. Taken in conjunction these two results establish that retinoic acid-induced EC cell commitment can proceed in the absence of any detectable inhibition of metabolic cooperation. This appears to preclude any essential role for the latter in the inductive process.

One explanation for the phenotype of PTmr0 is that the mechanism for induction of cell commitment is functional whereas a separate pathway for the effect on metabolic cooperation is defective. However, the responsiveness of PTmr0 towards the differentiation-inducing activity of retinoic acid is

approximately 10-fold less than that of its parental line PSA4TG12. The significance of this is uncertain. It could be a coincidence arising out of the selection procedure, as discussed in Section 3.4., but it also raises the possibility that the two effects of retinoic acid share a common mechanism, with different thresholds, which is reduced in sensitivity in PTmr0. Unfortunately it is not possible to determine whether the apparent resistance of junctional transfer is complete or represents only a decrease in sensitivity, since 10^{-4}M approaches the limit of solubility of retinoic acid in culture medium. Whether the two phenotypic changes are causally related or merely coincidental can only be formally established by isolation of a revertant with normal communication properties (see Section 3.4.). The ouabain rescue data on PC13.5 (Section 4.2.), however, indicate that the effects of retinoic acid on metabolic cooperation and cell commitment are differentially affected by feeders, which suggests that they are independent (Section 4.6.).

Unfortunately the biochemical studies on PTmr0 (Section 3.3.) shed little light on this issue as the lesion or lesions have not been identified. The variant contains comparable levels of CRABP to the parental line, consistent with mediation of the inductive actions of retinoic acid via this receptor (though this is evidently not the site of reduced sensitivity). There is no direct evidence for involvement of CRABP in the inhibition of metabolic cooperation. The limited data available on structural specificity for the latter (Pitts *et al*, in press, see Section 1.8.) are consistent with mediation by CRABP, but the concentrations of retinoic acid required far exceed those necessary for saturation of the binding protein (K_d=20-40nM) and cells apparently accumulate retinoic acid to a concentration exceeding that in the extracellular environment (Lotan *et al*, 1981). Moreover, the rapidity of the metabolic cooperation response precludes the involvement of *de novo* synthesis, as envisaged in the steroid hormone model (see Section 1.5.2.). These considerations suggest that CRABP does not mediate the effect of retinoic acid on junctional communication and the presence of functional receptor in PTmr0 cytosols is consistent with this. Lack of involvement of CRABP could be confirmed by a demonstration that retinoic acid blocks communication in cells which do not possess the binding protein. It is unfortunate in this regard that the two variants selected in Section 4.3. for non-responsiveness to retinoic acid both express CRABP.

It is also shown in Section 3.3. that PTmr0 exhibits a similar rate of cellular uptake of retinoic acid to PSA4TG12 and that it displays no gross alterations in

membrane properties. Possible explanations for the altered communication phenotype not directly examined in this study include the possession of an altered gap junction protein or a modified lipid domain associated with the junctional channel which renders metabolic cooperation in PTmr0 resistant to the action of retinoic acid, direct or indirect. A direct effect would clearly be mechanistically unrelated to the induction of cell commitment. This is not neccessarily the case for an indirect effect, however. For example, it is possible to conceive of small changes in the activity of membrane ion pumps which could modulate the processes of commitment and differentiation whilst larger changes might affect junctional permeability.

Although the present study has not elucidated the mode of action of retinoic acid in either inhibition of metabolic cooperation or induction of EC cell differentiation, PTmr0 may yet prove valuable in this respect. The question of a mechanistic connection between the two actions could be investigated at the cellular level via the techniques of somatic cell genetics and at the biochemical level by characterisation of the gap junction protein of the variant. It would also be of interest to determine whether PTmr0 is resistant to inhibition of metabolic cooperation by octanol. The latter is an amphipathic molecule, like retinoic acid, which has been shown to block junctional transfer in a variety of cell types (Johnston *et al*, 1980; Deleze and Herve, 1983; Bernardini *et al*, 1984). If metabolic cooperation in PSA4TG12 and other EC cells is sensitive to the inhibitory action of octanol but junctional communication in PTmr0 is unaffected, it would indicate that octanol and retinoic acid share a common mechanism, as suggested by Pitts *et al* (in press). Since differentiation of PC13.5 EC cells is not induced by incubation in medium supplemented with octanol (unpublished observations), this would imply that the mechanism of inhibition of metabolic cooperation is separate from that for induction of EC cell commitment.

The selection of two variant EC clones which do not differentiate on treatment with retinoic acid (Section 4.3.) adds to the two mutants previously isolated by Schindler *et al* (1981) and to a number of similar variants reported since the inception of this study (McCue *et al*, 1983; Jones-Villeneuve *et al*, 1983; Gudas and Wang, 1984; Moore *et al*, 1985). The lines obtained in the present study are derived from PC13.5 (Hooper and Slack, 1977). Both posses CRABP, as do the majority of variants described by other workers (see Section 1.5.2.), and their lesions remain unknown. The stability of the variant phenotype in the absence

of retinoic acid has not been investigated for either clone and there is no evidence as to whether they represent genetic modifications or epigenetic adaptations. The two clones appear to differ in their responsiveness to retinoic acid. PRA0.1, which was isolated without prior mutagenesis, does undergo some initial differentiative changes whereas PRA7c seems completely unresponsive (Section 4.3.2.). This suggests that the cellular mechanism of interaction with retinoic acid may be defective in PRA7c whilst it is the machinery of differentiation activated by this interaction which is deficient in PRA0.1. These two variants may be useful in investigations of the mechanism of retinoic acid action and the pathway of cytodifferentiation respectively. For example, it might prove instructive to compare their protein synthesis patterns by gel electrophoresis with those of PC13.5, both before and after exposure to retinoic acid. The response of specific activities believed to be important in the processes of commitment and/or differentiation, such as protein kinases (Section 1.5.2.), could also be examined.

It has previously been reported that several EC lines tested which differentiate when treated with retinoic acid also express CRABP (Section 1.5.). This finding,which provides circumstantial support for the hypothesis that retinoic acid-induced differentiation is mediated by CRABP, is extended by the present study to the feeder-independent line PC13.5. It is also shown for the first time that the feeder-dependent derivatives of PSA4 both undergo differentiation when exposed to retinoic acid and possess CRABP. In fact the demonstration that PSA4, and more significantly the EK line B2B2, are induced to differentiate by exposure to low concentrations of retinoic acid (in BRL-medium, Section 5.3.6.) strengthens the argument that this response is an appropriate model of differentiation events *in vivo* (though it does not necessarily imply that retinoic acid functions in this way in embryogenesis, see Sections 1.5.2. and 5.4.).

The cooperation-defective variant PT2md1 also possesses CRABP, so the unusual effect of retinoic acid on this EC line could be mediated via the cellular binding protein. The phenotype of PT2md1 is unique (Sections 4.5. and 4.6.). It appears to have almost completely lost the capacity for differentiation and is killed when exposed to chemical inducers such as retinoic acid. There is no evidence that the latter effect is related to the mec^- phenotype but it does seem to be associated with the differentiation-defective (dif^-) character (see below). The dif^- phenotype of PT2md1 is probably an independent consequence of the selection procedure, as discussed in Sections 4.5.5. and 4.6., and by

Smith (1984).

The high mortality of PT2md1 in the presence of chemical inducers, and its reduction in conditioned media known to inhibit differentiation indicate that the processes of cell commitment and cell death may be closely related in this cell line. Several suggestions are made in Section 4.6. for further investigations which should circumvent the technical difficulties in estimating induced differentiation encountered in the present study.

The inducing agents do not appear to be directly cytotoxic to undifferentiated stem cells, as witnessed by the increased proportion of stem cells and reduced mortality found in conditioned media (though the possibility that the conditioned media have two separate effects cannot be excluded, Section 4.5.4.). Moreover, there is evidence that the lower but still significant incidence of cell death found in other EC cell lines on exposure to retinoic acid is a response to the induction of differentiation (Mummery *et al*, 1984; see Section 4.5.4.). The association of cell death with commitment in PT2md1 therefore probably arises from either the execution of a lethally defective process of cellular differentiation, or the promotion of a normal developmentally programmed fate. Both are of considerable intrinsic interest but particularly the latter, since there are suggestions that cell death may play an important role in various aspects of embryogenesis, as discussed in Section 4.5.4. They cannot be distinguished on the available evidence, however. It is suggested (Section 4.6.) that time-lapse film analysis would be indispensable in this regard as a means of identifying the stage at which PT2md1 cells die and elucidating whether their death is determined in previous generations. In either case biochemical changes in PT2md1 following exposure to retinoic acid merit investigation. One method of doing this would be by analysis of protein synthesis patterns by gel electrophoresis. This could also provide informative comparisons with similar studies on PRA0.1 and PRA7c proposed above.

It is also noted in Chapter 4 that serum has profound effects on both the differentiation of PSA4-derived cells and their susceptibility to retinoic acid-induced cell death. This is another area which might reward further study. It would be of interest to determine whether in fact distinct differentiated cell populations are obtained in the presence of different sera and if the composition of these populations is affected by prior exposure to retinoic acid or HMBA (Section 4.6.). The object of such an investigation would be to

discover to what extent it may be possible to reproducibly direct the differentiation of pluripotent EC or EK cells along diverse developmental pathways in monolayer culture (see also the discussions on retinoic acid-induced differentiation in Section 5.4. and below). Hopefully this might enable elucidation of the nature of some of the factors which modulate the process of cell determination, particularly as serum substitutes are now available which contain only small amounts of undefined constituents.

During the course of this study various agents, both purified chemicals and media conditioned by different cell types, were examined for their ability to induce or inhibit the differentiation of either PC13.5 or PSA4 EC cells. No novel activities were identified until the employment of medium conditioned by Buffalo rat liver (BRL) cells (Chapter 5). This was found to have a dramatic effect on PSA4 and its derivatives, completely preventing the spontaneous differentiation of these pluripotent EC cells which occurs when they are plated in the absence of a feeder layer. The action of BRL-conditioned medium is shown in Section 5.3. to be stable and reversible, to be independent of EC cell density, to entail no significant cell selection, to introduce no karyotypic abnormalities and to impose no restrictions on future developmental potential. It is effective on a variety of EC cells and also on the EK line B2B2. The effect of BRL-conditioned medium is far greater than that of STO feeder-conditioned medium previously reported from this laboratory (Smith and Hooper, 1983). It enables for the first time the long-term, feeder-free maintenance of homogeneous stem cell populations of pluripotent feeder-dependent EC and EK cells.

Such cells generally display greater developmental capacity than their feeder-independent counterparts (see Section 1.3.4.) and in the case of EK cells possess several other advantages (Section 1.6.), but their experimental manipulation hitherto has been limited to a considerable extent by the requirement for feeders. The major disadvantages imposed by feeders are:-

(a) Logistical constraints
(b) Restrictions on selective systems
(c) Heterogeneous cell populations
(d) Obscure cellular interactions
(e) Contamination with escaped feeders
(f) Possibility of DNA transfer

The logistical constraints stem from the necessity of constantly producing new feeder layers, as they are only fully functional for a few days. Moreover, in the case of some EK lines the feeders apparently have to be prepared from primary cultures of embryonic fibroblasts. Not only does this significantly increase the work involved in routine culture but it also multiplies the risks of contamination with micro-organisms. These problems are amplified during selection procedures which may require the maintenance of large EC or EK populations for prolonged periods.

The selection of variants, transfectants or hybrids can also be directly affected by the use of feeders. Selective agents may be toxic to the feeder cells causing rapid disintegration of the feeder layer. In theory this might be circumvented by employing cells bearing appropriate genetic markers as feeders. However, very few established cell lines make suitable feeder layers and the number of variants available is limited. Furthermore, if the feeder cells are resistant they may rescue sensitive EC or EK cells via metabolic cooperation (the "kiss of life", Section 1.8.). Thus it would not be possible to isolate a ouabain resistant EC clone on an STO feeder layer, for example. Conversely, resistant feeders could be disrupted by the junctional transfer of toxic metabolites from sensitive stem cells, as occurs in the thioguanine "kiss of death" procedure (see discussion on the isolation of PT2md1 in Section 4.5.4.). Finally, resistant feeder cells might permit the transfer of toxic chemicals or metabolites to otherwise resistant EC or EK clones.

The presence of feeders also interferes with biochemical and molecular studies by introducing heterogeneity into the cell population. Hence the majority of such work has been performed on feeder-independent lines, which may be more likely to exhibit abnormalities (see Sections 1.3. and 1.7.). Similarly the presence of feeders complicates the study of the properties of intact stem cells and their interactions with exogenous factors. The process by which feeders facilitate EC and EK cell survival and proliferation is complex, involving both contact-independent and contact-dependent mechanisms (Isacke and Deller, 1983; see also Chapter 7) and it is not clear to what extent these, or other activities of the feeders, modulate the effects of exogenous agents such as retinoic acid. Furthermore it is difficult to detect, let alone analyse, the occurrence of differentiation on a feeder layer background.

Feeder cells are growth-arrested by X-irradiation or by incubation with

mitomycin C. However, there is a finite probability of a cell escaping this treatment and continuing to proliferate. If EC or EK cultures become contaminated with such escaped feeders, they can only be rescued by re-cloning.

Finally, as discussed in Section 5.4., there is always a theoretical possibility of DNA being transferred from feeder cells to stem cells. This is particularly relevant in the case of EK cells where it is important to establish that the stem cells have not been transformed.

The use of BRL-conditioned medium certainly overcomes the first five of these difficulties and may also surmount the sixth. It thus represents a considerable experimental advance in the use of pluripotent EC and EK cells as model systems for the analysis of murine embryogenesis. Possible applications include:-

(a) Selection of variants
(b) Potential isolation of EK cells in the absence of feeders
(c) Generation of homogeneous populations for biochemical and molecular studies
(d) Investigation of interactions with chemical inducers and other exogenous factors
(e) Development of defined media for EK cells
(f) Analyses of cell determination and differentiation
(g) Embryo culture -potential experimental and biomedical applications

Points (a) to (d) follow directly from the preceding discussion. [Examples of (d) are provided by the study of the effects of retinoic acid on PTmr0 and PT2md1 reported in Chapters 3 and 4 respectively.] The fifth suggestion is an extension of the preceding point and could prove significant in determining whether the proliferation of embryonic stem cells is subject to regulation by exogenous factors (see discussion in Section 1.7.). The final proposal is speculative and, as with (e) is dependent on purification of the "Differentiation Inhibiting Activity" (DIA) (see below).

Some of the potential applications of DIA to the study of cell determination and differentiation are illustrated by the results presented in Sections 5.3.4. and

5.3.6. These indicate that differentiation, induced by aggregation and retinoic acid treatment respectively, is restricted to extraembryonic endoderm in BRL-conditioned medium. This suggests that the inhibitory effect of DIA is selective. Specifically, it may permit commitment to the extraembryonic endoderm lineage but block determination to other lineages (Section 5.4.). Spontaneous differentiation of PSA4 or B2B2 in normal medium in the absence of feeders is to a different cell type(s) (Section 5.3.6.). Both retinoic acid-induced differentiation in BRL-conditioned medium and spontaneous differentiation in CM occur throughout the stem cell population. Consequently it should be feasible to compare the two processes at the biochemical and molecular levels, which may enable identification of the key steps in cell determination.

The apparent lineage restriction of the action of DIA can be interpreted in the context of events in normal embryogenesis, as discussed in Section 5.4. Such an interpretation implies that DIA may be functional in the early embryo. This proposition is strengthened by the finding that a similar activity is present in medium conditioned by the PYS parietal endoderm cell line, though not by PC13.5 EC cells or PSA5E visceral endoderm cells (Section 6.3.2.). This indicates that DIA could be produced by parietal endoderm *in vivo* and thus be present in the implantation stage embryo. Whether it might also be produced by promitive endoderm is uncertain, but seems probable given that primitive endoderm apparently expresses many other functions and markers of parietal endoderm plus some of those of visceral endoderm (Kahan and Adamson, 1983; Hogan *et al*, 1983; Grabel *et al*, 1983).

The possible production of DIA by primitive and parietal endoderm but not by visceral endoderm can be rationalised in terms of the development of the egg cylinder (Section 1.2.2.). Its effects on embryoid bodies (Section 5.3.4.) suggest that the primary function of DIA may be to maintain an undifferentiated inner cell mass population until after implantation. If this is the case, there is no requirement for DIA production by visceral endoderm. On the contrary, it is expected that visceral endoderm would not produce DIA. Then, following implantation, as the primitive endoderm overlying the epiblast undergoes maturation into visceral endoderm and the parietal endoderm migrates over the trohoblast (Fig.1.1.), the level of DIA in the primitive ectoderm would fall, allowing pro-amniotic cavity formation and further differentiation.

Such a model predicts that DIA is produced in large amounts by primitive endoderm and that the stimulus for differentiation of the epiblast is withdrawal of DIA rather than production of some inducer. This hypothesis is consistent with observations on embryoid bodies which indicate that endoderm maturation may be a prerequisite of cavitation (Table 8.1).

Table 8.1
Cavity Formation by Aggregates of EC Cells Under Various Conditions

Cell Line	Medium Supplement	Endoderm* PrE	PE	VE	Cavity Formation	Ref.
PSA4	-	+	+	+	+	1
PSA4	DIA	+	+	-	-	2
S2	-	+	+	-	-	1,3
PSA1	-	+	+	+	+	1
PSA1	Tunicamycin	+	-	-	-	4
F9	RA[a]	+	+	+	+	5,6
F9	RA+dbCAMP[b]	+	+	-	-	5,6
F9	Laminin	+	-	-	-	6

*PrE, primitive endoderm; PE, parietal endoderm; VE, visceral endoderm
[a] Retinoic acid
[b] Dibutyryl cyclic AMP
References:-
1. Martin and Evans (1975a)
2. Section 5.3.4. above
3. Martin *et al* (1977)
4. Grabel *et al* (1983)
5. Hogan *et al* (1981)
6. Adamson and Grover (1983)

These observations have generally been interpreted as implying an inductive role for visceral endoderm in the process of cavity formation (Martin *et al*, 1977; Uno; 1982). However, the absence of the visceral phenotype from the endoderm layer corresponds to the presence of increased amounts of the parietal form and/or the persistence of primitive endoderm and consequently could equate to high levels of DIA production. For example, the failure of embryoid bodies formed by EC lines such as S2 to undergo cavitation and further differentiation *in situ* (Martin and Evans, 1975a,b; Martin *et al*, 1977) may arise not from their failure to develop visceral endoderm *per se*, but rather from their relative over-production of parietal endoderm and/or the possible persistence of primitive endoderm. This may generate a high local concentration of DIA in the core of the embryoid body, thus preventing cavitation and further differentiation. On attachment, this microenvironment

may be dissipated due to migration of the endoderm cells off the embryoid bodies onto the substratum and/or to the breakdown of the basement membrane which physically constricts the EC core in suspension culture. Consequently differentiated outgrowths develop (Martin and Evans, 1975c). Such differentiation is of course inhibited in BRL-medium (Section 5.3.4.) which contains high levels of DIA.

It is difficult to distinguish experimentally between the alternatives of inhibitory control by primitive/parietal endoderm and inductive regulation by visceral endoderm. One approach which might prove informative would be to form mixed embryoid bodies between PSA4 and PYS-2 and determine whether cavitation can be inhibited, by increasing the contribution of parietal endoderm. It is also clearly important to establish whether visceral endoderm is present in the non-cavitating embryoid bodies formed in BRL-conditioned medium.

Ultimately, however, the DIA hypothesis advanced here could only be confirmed by demonstrating that the same activity is produced by primitive and parietal endoderm cells during normal embryogenesis. This requires purification of DIA and the subsequent development of immunological and *in situ* hybridization assays. Pure DIA is also required for some of the experimental applications mentioned above, not least because it is known that BRL cells produce at least two other growth factors, IGF-II and TGF-β, which may interact with embryonic cell types. Purification is in fact essential for determining the relationship of DIA to growth factors and oncogene products and confirming whether, as is suggested by the evidence presented in Chapters 5 and 6, it represents a novel type of activity involved in the negative control of cellular differentiation and embryonic development.

Unfortunately only limited purification has been achieved in the present study (Section 6.2.) This reveals that DIA is an acid-stable, relatively heat-stable, entity of apparent molecular weight 20-35,000. It may be associated with a mitogenic acitvity but does not correspond to any of a series of known growth factors tested, both individually and in combination (Section 6.3.). Purification to homogeneity and amino acid sequencing are therefore required. If the activity is a previously unidentified species, it is of obvious importance to then develop a gene clone.

Finally, the fact that DIA is produced by mature liver cells suggests that its

actions may not be confined to the embryonic epiblast. An activity produced by BRL cells which inhibits myogenesis has been described (Evinger-Hodges *et al*, 1982). It would therefore be of interest to examine the effects of DIA on myoblast differentiation. Another system worthy of attention is the differentiation of myeloid stem cells. Like EC cell lines, the human promyelocytic leukaemia line HL60 undergoes terminal differentiation when exposed to retinoic acid. The effect of DIA on HL60 differentiation might reward investigation.

The investigation in Chapter 7 into the role of 2-mercaptoethanol demonstrates that this thiol compound is essential for viability of PSA4 EC cells in the absence of feeders, but that it is unlikely to have any direct effect on differentiation or to interact with DIA (Section 7.2.). A possible mode of action of DIA is proposed in Section 7.4. This predicts that mercaptoethanol-dependent EC and EK cells lack a particular enzyme activity, methylthioadenosine nucleoside phosphorylase (MTANP), which renders them dependent on an exogenous source of methyl mercaptan. This hypothesis is amenable to experimental test as indicated in Section 7.4.

It is also consistent with the preliminary findings reported in Section 7.3., which indicate that feeders may rescue dependent cells in the absence of 2-mercaptoethanol via transfer of some metabolite through gap junctions. Further experiments are needed to substantiate this argument. Junctional transfer of a factor or factors required for cell division, as methyl mercaptan appears to be (Toohey and Cline, 1976), could account for previous reports that contact-dependent mechanisms are involved in the promotion of EC cell growth by feeders (Isacke and Deller, 1983). It also raises the possibility that junctional communication with differentiated cell types may be necessary for maintenance of embryonic stem cells *in vivo*.

The interactions of retinoic acid with EC cells have provided the subject for much of the experimental work in this thesis. Unfortunately, the complex nature of the cellular responses has hindered investigation of their interrelationships and few conclusions can be drawn with any degree of certainty. Further experimental approaches are suggested and it is possible that PT2md1 and the two new variants, PRA0.1 and PRA7c, may prove useful tools in the analysis of retinoid effects and the study of commitment and cellular differentiation. The major contribution of this thesis, however, is the finding that medium

supplemented with 2-mercaptoethanol and a stable "Differentiation Inhibitng Activity" produced by rat liver cells can replace feeder layers in the maintenance of undifferentiated populations of EC and EK cells. This represents a considerable experimental advance, some of the possible applications of which have been indicated. The discovery of DIA also has theoretical implications, since it indicates that the differentiation of embryonic stem cells may be actively suppressed and consequently implies that the normal tendency of these cells may be to differentiate, not to proliferate continuously as has been argued elsewhere (Heath, 1983; see Section 1.7.). The actions of DIA could therefore be representative of a system of negative control in embryonic development. Clearly the purification and molecular characterisation of this activity merit attention and may yield new insights into our understanding of developmental processes.

I. APPENDIX

CALCULATION OF THE APPROXIMATE ORDER PARAMETERS OF NITROXIDE SPIN LABELS IN PHOSPHOLIPID BILAYERS

Electron spin resonance spectroscopy detects transitions, induced by electromagnetic radiation, between spin states of unpaired electrons in a magnetic field (see Knowles *et al*, 1976, for a detailed discussion of magnetic resonance spectroscopy). In practice, the electromagnetic frequency is held constant and the magnetic field is varied. The lines in an ESR spectrum can be split by interaction of the electron magnetic moment with the nuclear magnetic moment. This hyperfine splitting is determined by the nuclear spin number, I. For nitroxide spin labels such as the doxyl group, the nitrogen N^{14} nucleus gives rise to three hyperfine lines in the spectrum (Fig.A1). It is the anisotropy of this hyperfine splitting with respect to the magnetic field direction which confers structural and motional sensitivity on the spin label technique.

The spectrum of a nitroxide radical tumbling freely in aqueous solution has a uniform splitting and the resonances are of equal intensity (Fig.A1c). In contrast, a spin label reporter group on a fatty acid incorporated into a phospholipid bilayer is partially immobilized. This renders the hyperfine splittings anisotropic. Maximum hyperfine splitting ($A_{||}$) occurs with the magnetic field parallel to the membrane normal, and minimum splitting ($A_{\perp}$) is obtained when the field is perpendicular to the normal (Knowles *et al*, 1976). However, the spin label undergoes rotational motion about the long axis of the fatty acyl chain. This limited anisotropic motion, restricted by the segmental flexibility of the spin-labelled molecule and the fluidity of the bilayer, averages out the overall anisotropy of the spectrum. The extent of this motional averaging is given by the order parameter, S

$$S = \frac{A_{||} - A_{\perp}}{A_{zz} - 0.5(A_{xx} + A_{yy})}$$

where A_{zz},A_{xx} and A_{yy} are the principal values of the hyperfine tensor measured when the spin label is totally immobilized in a single crystal host. The order parameter is simply the ratio of the observed hyperfine anisotropy to the maximum theoretically obtainable i.e. completely rigid orientation.

Fig.A1 ESR Spectra of a Nitroxide Spin Label Under Various Conditions

Adapted from Knowles *et al* (1976).

Plate 5.6 Embryoid Bodies Formed From PSA4 After Feeder-Free Culture in BRL-Medium

PSA4 cells, previously passaged 4 times in BRL-medium, were induced to form embryoid bodies via aggregation in EfC10 as described in Section 2.10.3. Embryoid bodies were fixed in Bouin's fluid after 4 or 6 days of suspension culture and histological sections stained with haemotoxylin and eosin.

	Magnification
A. 4 day embryoid bodies	66
B. 6 day embryoid body	74
C. Blood islands in 6 day embryoid body	165
D. 4 day embryoid bodies	82.5
E. 4 day embryoid body	186

Plate 5.7 Metaphase Chromosome Spread of PSA4 After More Than 40 Generations of Feeder-Free Culture in BRL-Medium

Magnification: 1032

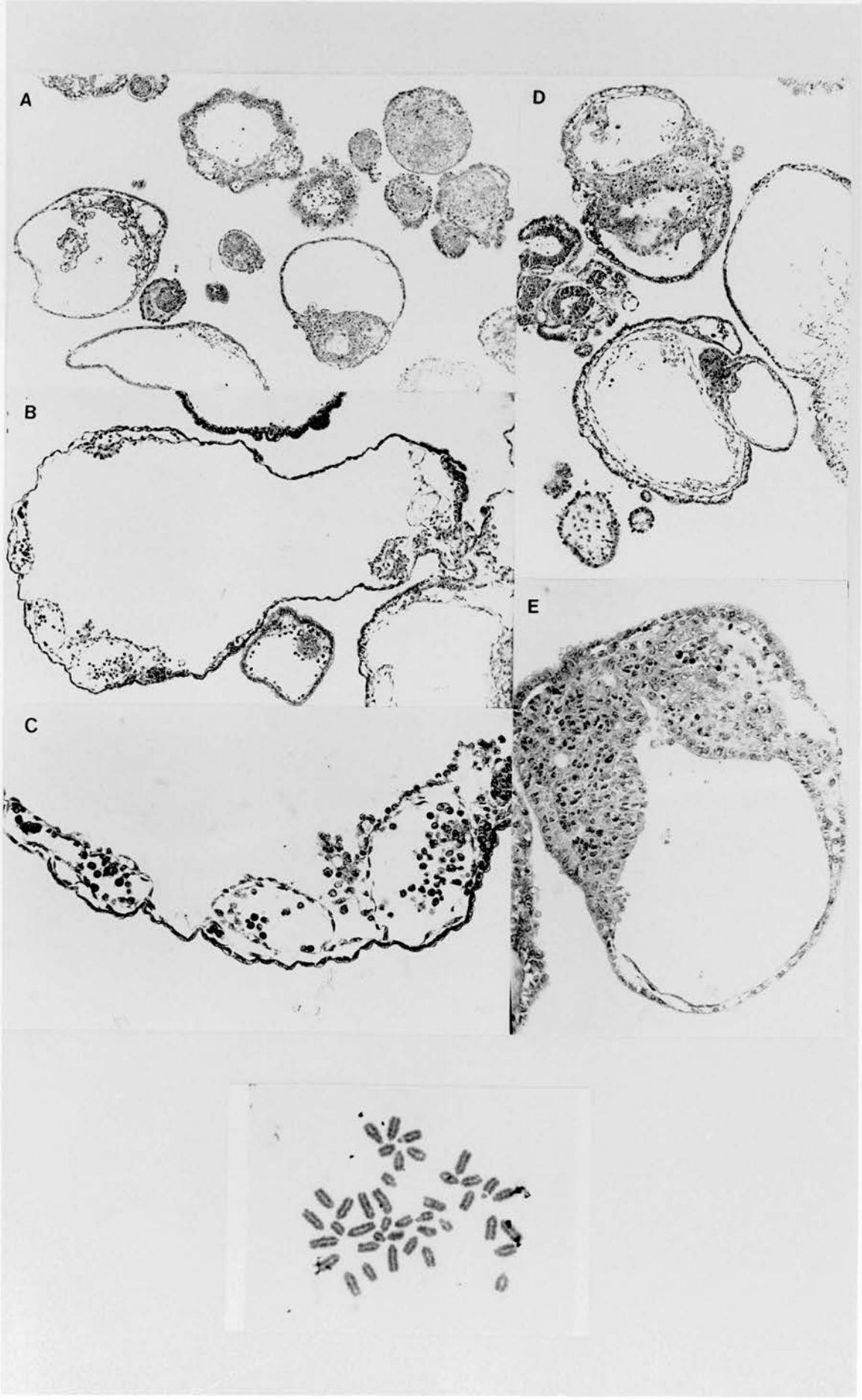
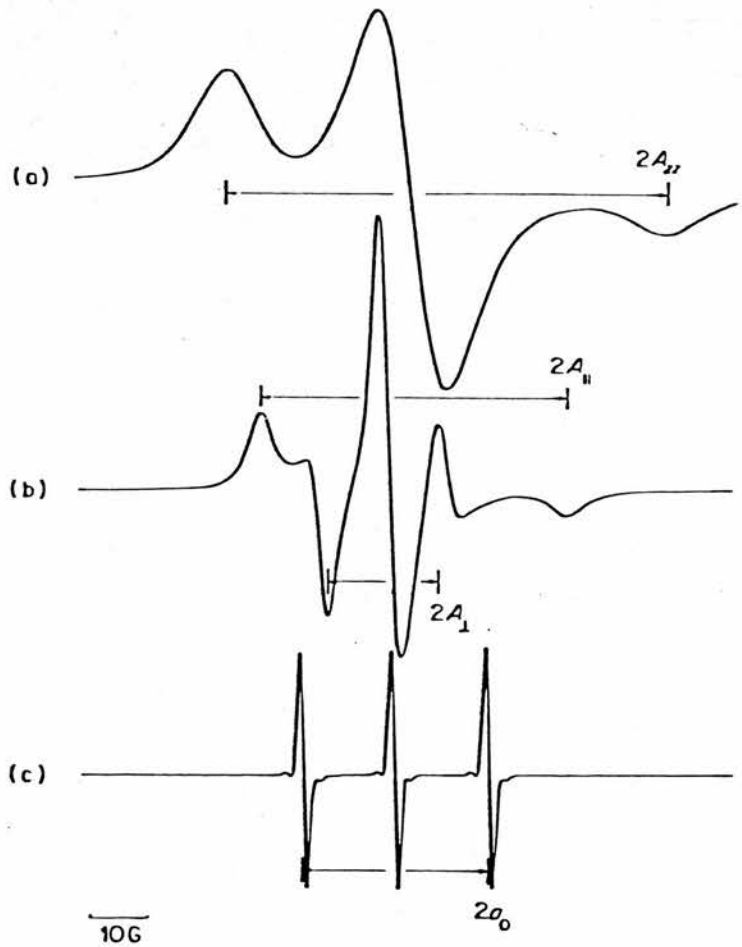

A
B
C
D
E

Fig. A1 ESR Spectra of a Nitroxide Spin Label Under Various Conditions



Anistropy of a nitroxide spin-label ESR spectrum under various conditions of motion. (a) Powder spectrum, from a nitroxide randomly and rigidly oriented in frozen solution. (b) Lipid dispersion spectrum, from a nitroxide spin label undergoing anisotropic motion in a randomly oriented lipid dispersion. (c) Isotropic spectrum, nitroxide randomly tumbling in a non-viscous solution

Order parameters can be calculated from experimental spectra (Berliner, 1976) by assuming that $2A_{||}=2A_{max}$, where $2A_{max}$ is the separation of the outer extrema of the spectrum, and $2A_{\perp}=2A'_{min}$, where $2A_{min}$ is the separation of the inner extrema (see Fig.A1b). With these assumptions, and ignoring any difference in polarity between the single crystal and the experimental environment, the approximate order parameter, S_{app}, is given by

$$S_{app} = \frac{A_{max}-A_{min}}{A_{zz}-0.5(A_{xx}+A_{yy})}$$

Substituting the principal values of the doxyl hyperfine tensor (Berliner, 1976) gives

$$S_{app} = \frac{A_{max}-A_{min}}{27.55}$$

where all values are in gauss (G).

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PUBLICATIONS

The following has been submitted to Experimental Cell Research:-

Smith,T.A., Smith,A.G. and Hooper, M.L.
Selection of a mouse embryonal carcinoma clone resistant to the inhibition of metabolic cooperation by retinoic acid